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LABORATORY DIAGNOSTIC METHODS

LABORATORY DIAGNOSTIC METHODS

PATHOLOGICAL, BACTERIOLOGICAL, SEROLOGICAL AND CHEMICAL

A MANUAL FOR PHYSICIANS, MEDICAL STUDENTS AND LABORATORY TECHNICIANS

BY

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PREFACE

As indicated by the title "Laboratory Diagnostic Methods," this book aims simply to present in a concise manner the technic of various laboratory tests without any discussion of their value, limitations or clinical applications in the diagnosis and treatment of disease. The style adopted is the same as that used originally by Pepper and Klaer in their little *Manual on Clinical Pathology,* which was revised and re-edited about twelve years ago by Pepper and one of us (J. A. K.) for use in the Medical School of the University of Pennsylvania. Dr. Pepper has kindly given his consent to the revision of the *Manual* and its inclusion in the first section of the present book devoted to Clinical Pathological Methods. The little book has had such a wide appeal and has proved so helpful to physicians, medical students and laboratory technicians, that we have been urged to revise and enlarge it by including methods used in clinical bacteriology, serology and blood chemistry.

We hope that the present book will fulfill the requirements and meet with the expectations and requests of numerous physicians, laboratories, teachers and technicians. It represents essentially the clinical, pathological, bacteriological and serological methods employed in the Department of Pathology and Bacteriology of the Graduate School of Medicine in the University of Pennsylvania and its Medico-Chirurgical and Polyclinic Hospitals. The book is especially designed for busy practitioners who desire brief, yet comprehensive descriptions of various tests and methods. We also hope that it will prove helpful to pathologists, bacteriologists and laboratory technicians in general as well as to students of medicine.

Histological methods and the technic of the preparation of culture media are not included, as the methods employed by us in the laboratories of the Graduate School of Medicine are described

in a separate little manual, *Practical Laboratory Methods and Histologic Technic,* by Miss Elizabeth Arthur, and published by the F. A. Davis Company.

Illustrations have been purposely omitted; satisfactory ones are usually to be found in the catalogues of firms supplying laboratory apparatus and materials. It is assumed that those who use our book have had some experience in laboratory work and that beginners will have the guidance of more experienced workers.

We beg to express our thanks to Dr. H. L. Bockus, who kindly reviewed the sections on gastric analysis, the examination of bile and the examination of feces; also to Miss Elizabeth M. Yagle, for assistance in arranging a portion of the section on Clinical Serological Methods.

John A. Kolmer
Fred. Boerner.

Philadelphia.

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SECTION I

PATHOLOGY

SECTION I

CLINICAL PATHOLOGICAL METHODS

EXAMINATION OF THE BLOOD

Method of Cleaning Blood Pipets

1. Draw water through pipet until all traces of blood and serum are removed.
2. Without drying draw through alcohol or acetone.
3. Draw through ether.
4. Draw air through until the pipet is dry (if properly dried the bead in the bulb should be freely movable).

All pipets should be cleaned immediately after use.

If blood has dried in the stem of a pipet remove it with horse hair or fine stiff wire and fill pipet with antiformin or nitric acid and allow to stand over night; then clean thoroughly as described.

Methods for the Estimation of Hemoglobin

Sahli Method

1. Fill the graduated tube of Sahli's hemometer with n/10 solution of hydrochloric acid to the mark 10.
2. After cleansing finger tip or lobe of ear with alcohol, make a puncture with lancet and gently compress (*the blood should flow freely*); wipe off first drop, then fill pipet to the mark 20 with blood.
3. Immediately place the blood in the graduated tube containing the HCl solution. Remove the last trace of blood by drawing the solution up in the pipet and expelling several times.
4. Dilute the mixture in the graduated tube with water until it has the same tint and intensity of color as the standard tube. This

is best done by adding a few drops at a time and comparing after each addition.

5. When both tubes have exactly the same tint and intensity note the figure at the height of the graduated tube. This figure represents the percentage of hemoglobin.

In some instruments the normal for men is 80 and 70 for women. The corrected percentage of hemoglobin is obtained by $\frac{H}{N}$; H = per cent obtained and N the normal. For example, if the reading is 60 for a male patient, the correct estimate of hemoglobin would be $\frac{60}{80} = 75$ per cent; for a woman it would be $\frac{60}{70}$ or 86 per cent.

Dare Method

1. See that the porcelain and glass slides of the Dare hemoglobinometer are perfectly clean and tightly clamped in holder.

2. Screw the camera tube into place and adjust candle or see that electric bulb is in working order.

3. Fill capillary space between glass plates by placing one corner in contact with a large drop of blood.

4. Wipe off excess of blood and place holder in proper position in instrument.

5. If candle is used the blue flame should be below the color aperture and wick not over half an inch in length.

6. Examine in a dark closet, or dark corner of room, moving color scale until it exactly corresponds to color of blood between the glass plates.

7. Read off the number indicated at the beveled edge of the rectangular opening of the case. This figure represents the percentage of hemoglobin.

Newcomer Method

1. Insert the Newcomer attachment [1] in the light path of one of the cells of a Duboscq colorimeter—at the top of plunger. This

[1] The A. H. Thomas Co., Philadelphia, supply this special glass, cut, ground and polished to thickness, the thickness being engraved on each piece. They also supply the necessary pipets, and the "daylite" unit for use at night consisting of an 8″ Rounded of "daylite" glass and an appro-

same attachment may be used in the Bock-Benedict [2] colorimeter, in which case it is placed below the mirror. *Note the thickness* [3] marked on the glass plate attachment before inserting.

2. Fill the corresponding cup with water.

3. Place 5 c.c. of decinormal (n/10) HCl in the other cup.

4. Puncture lobe of ear or finger in same manner as described for cell counting and collect 20 c.mm. of blood in a capillary pipet.

5. Place the blood in the cup containing the n/10 HCl and *note the time.*

6. Balance the two fields of the colorimeter by lowering the plunger in the cup containing the blood.

7. Make reading and *note the time.*

8. In the *calculation* the hemoglobin reading in gm. per 100 c.c. of whole blood is represented by the quantity $\frac{0.38d}{t}$ where d is the dilution (in this case 251); t, the thickness of the balancing solution in mm., the colorimeter reading; and 0.38 a number empirically determined for a 1 mm. glass.[4] This expression has to be modified because of the fact that the hematin does not reach its full density for some time. If x is the time in minutes after making the dilution from the pipet, then the reading is too small by a percentage equal to $\frac{40}{x}$ and the real reading is

$$\frac{38d}{+(100=\frac{40}{x})}.$$

It will be seen that when x is very large this becomes equivalent to the first formula given. When x is equal to 10 (at the end of 10 minutes), the hematin has reached to within 4 per cent, in 20 minutes to within 2 per cent, and in 40 minutes to within

---

priate shade. The measured glass discs will also be provided as a part of the regular equipment of the new Bausch and Lomb colorimeter of the Duboscq type.

[2] The colorimeter described by J. C. Bock and S. R. Benedict is well adapted for this purpose. The colorimeter cup is small enough to be almost filled by 5 c.c. of solution, thus permitting readings to be made on bloods running as low as 20 per cent of the normal without its being necessary to increase the density of the hematin solution by using a larger amount of blood. For the ordinary Duboscq cup the 20 c.mm. of blood suffices for hemoglobin readings down to 50 per cent of the normal.

[3] See tables I and II for correcting thickness of glass attachment.

[4] Methods for calculation are taken from Newcomer's original article.

1 per cent of its final value. For mental calculations the figure given by the simple formula $\frac{0.38d}{t}$ may therefore be simply increased by these percentages.

Tables I and II give in convenient form the figures into which t, the thickness of the balancing solution in mm., is to be divided in order to obtain the concentration of hemoglobin in gm. per 100 c.c. of whole blood and in per cent Haldane. The dilution is taken as 251. To each of certain possible thicknesses of colored glasses there corresponds a column of figures corrected for the time factor in the formation of acid hematin.

To obtain gm. of hemoglobin per 100 c.c. of whole blood divide the colorimeter reading into the figure of the appropriate square in table I, the choice of the square depending upon the thickness of the glass used and the length of time since dilution of the blood was made.

TABLE I

Time since Dilution in Minutes	Thickness of Colored Glass in Millimeters										
	0.95	0.96	0.97	0.98	0.99	1.00	1.01	1.02	1.03	1.04	1.05
10.....	94.4	95.4	96.4	97.4	98.4	99.4	100.4	101.4	102.4	103.4	104.4
15.....	93.1	94.1	95.0	96.0	97.0	98.0	99.0	100.0	101.0	102.0	103.0
20.....	92.5	93.5	94.5	95.4	96.4	97.4	98.4	99.4	100.4	101.3	102.3
30.....	91.8	92.8	93.8	94.8	95.7	96.7	97.7	98.6	99.6	100.5	101.5
40.....	91.6	92.5	93.5	95.5	95.4	96.4	97.4	98.3	99.3	100.3	101.2
Final.	90.6	91.6	92.5	93.5	94.4	95.4	96.4	97.3	98.3	99.2	100.2

To obtain the per cent hemoglobin Haldane scale, divide the colorimeter reading into the figure of the appropriate square, choice of the square depending upon the thickness of the glass and the length of time since dilution of the blood was made.

TABLE II

Time since Dilution in Minutes	Thickness of Colored Glass in Millimeters										
	0.95	0.96	0.97	0.98	0.99	1.00	1.01	1.02	1.03	1.04	1.05
10.....	683	690	697	704	712	719	726	733	740	748	755
15.....	673	680	687	694	702	709	716	723	730	737	744
20.....	669	676	683	690	697	704	711	718	725	732	739
30.....	664	671	678	685	692	699	706	713	720	727	734
40.....	662	669	676	683	690	697	704	711	718	725	732
Final..	665	662	669	676	683	690	697	704	711	717	724

Enumeration of Erythrocytes

1. After cleansing finger, or lobe of ear (preferred), with alcohol and allowing to dry, puncture tip and wipe off first drop of blood. Avoid excess squeezing of the finger (if the fingers are cold they should be rubbed briskly or immersed in hot water for a few minutes and dried).

2. Draw blood up to exactly the 0.5 mark of the pipet marked 101.

3. Immediately draw up diluting fluid to the mark 101, thus making a dilution of 1-200. Use either 0.85 saline solution or Hayem's solution for diluting.

4. Rotate pipet well for several minutes, holding in a horizontal position and finally shake sidewise.

5. Place cover slip in position on counting chamber (Neubauer ruling). Both cover slip and slide should be clean and free of lint or dust particles which may interfere with their proper apposition.

6. Blow out two or three drops from the pipet and then place a small drop in space under cover slip. By placing the

drop at edge of the space the chamber fills by capillarity. Avoid overfilling.

7. Allow a few minutes for the corpuscles to settle.

8. Examine with 1/6 or 2/3 objective.

9. The counting slide, when examined under the microscope, will be found to have a number of small squares marked upon it. The size of these small squares is 1/20 mm. by 1/20 mm. or 1/400 sq. mm. When the cover slip is in place there is a chamber formed measuring 1/10 mm. in depth. Therefore the small squares are in reality cubes measuring 1/4000 c.mm. ($1/20 \times 1/20 \times 1/10$ mm. $= 1/4000$ c.mm.).

It is impracticable to count the number of cells in 4000 of these small cubes which would give the number of cells per c.mm. of diluted blood, so that a smaller number of cubes should be chosen.

10. Count the number in 80 of the small squares. It will be noted that the small squares are separated into groups of sixteen. Five of these groups therefore contain 80 small squares. Do not count those cells touching the lower and right-hand lines.

11. Divide the number of cells found in 80 small squares by 80, thus determining the average number per small square or in 1/4000 c.mm.

12. Multiply the average number of cells per 1/4000 c.mm. by 4000 to determine the number of cells in 1 c.mm. of diluted, and by 200 to determine the number of cells in 1 c.mm. of undiluted blood.

The above described calculation can be thus represented: Let x = the number of cells counted in 80 small squares. Then x divided by 80 equals the number of cells in one small cube $\therefore$ x divided by $80 \times 4000 \times 200 = x \times 10{,}000$ = the number of cells in 1 c.mm. of undiluted blood. *Therefore in routine work count the number of cells in 80 small squares and add four ciphers.*

Preparation of 0.85 per cent solution of NaCl

Sodium Chlorid (C.P.)	0.85 gm.
Distilled water	100.00 c.c.

Add a crystal of thymol.

Preparation of Hayem's Solution

Bichlorid of mercury	0.5 gm.
Sodium sulphate	5.0 gm.
Sodium chlorid	1.0 gm.
Distilled water	200.0 c.c.

Estimation of the Color Index

1. Determined by dividing the percentage of hemoglobin by the percentage of red cells as compared with a recognized normal, namely, 5,000,000 red cells per 1 c.mm.

2. In routine work the color index may be determined by dividing the percentage of the hemoglobin obtained, by the first two figures of the red cell count multiplied by 2.

Estimation of the Volume Index

Method of Keith, Rowntree and Geraghty

1. Fit rubber tubing to the calibrated glass tubes of the Daland hematocrit.

2. Puncture the finger and fill both tubes with blood. At the same time fill blood pipet for making erythrocyte count.

3. Smear a little vaselin over tip (beveled end) and remove rubber tubing.

4. Place tubes in holder and centrifuge for three minutes at 10,000 revolutions per minute.

5. Read the percentage of red cells to whole blood directly from pipet. The 100 division marks represent the percentages.

6. Make erythrocyte count. The result is expressed in percentage taking 5,000,000 as a normal.

7. Divide the per cent of red cells by volume by the per cent of red cell obtained by counting. The result is the volume index.

$$\frac{\text{Percentage by volume}}{\text{Percentage by counting}} = \text{Volume index}$$

Enumeration of Leukocytes

1. After cleansing the tip of the finger or lobe of ear, puncture and secure drop of blood. Avoid undue squeezing; the blood should flow freely.

2. Draw blood up to exactly 0.5 mark on the pipet marked 1-11.

3. Draw up diluting fluid to mark 11, thus making a dilution of 1-20.

4. Rotate pipet well for several minutes, holding the pipet in a horizontal position; finally shake briefly sideways.

5. Blow out several drops.

6. Fill counting chamber (Neubauer ruling) and examine same as for erythrocyte count.

7. One of the following methods of counting and calculating may be carried out:

(*a*) Count the cells contained in the four corner units (each unit contains 16 small squares). Divide by 4 to obtain the number per unit (1 sq. mm.) and multiply by 200. The result represents the number in 1 c.mm. of undiluted blood.

(*b*) Count the cells in the four corner units and multiply the total by 50. The result represents the number of cells in 1 c.mm. of undiluted blood.

8. If other rulings are used obtain the number of cells per sq. mm. and multiply by 200.

Diluting fluid

Acetic acid	0.5 c.c.
Water	100.0 c.c.

Enumeration of Platelets

1. Prepare finger or lobe of ear and make puncture same as for erythrocyte count.

2. Remove first drop of blood. From the second drop fill the red corpuscle blood pipet marked 101 up to the mark 1.

3. Immediately fill pipet to the mark 101 with diluting fluid.

4. Mix well and fill counting chamber. Use special thin cover glass.

5. Allow to stand for 10 minutes before counting.

6. Examine with 1/6 objective.

7. Red cells appear as shadows, white cells have deeply stained nuclei, platelets appear as oval or round bodies stained a lilac color.

8. Count at least 200 small squares.

9. Calculation (see erythrocyte count page 9).

200 small squares = 1/20 c.mm. Therefore the total number counted multiplied by 20 = the number in 1 c.mm. of diluted blood. Then multiply this number by 100 to obtain the number in 1 c.mm. of undiluted blood. *In routine work multiply the total number of platelets counted in 200 squares by 2000.*

Diluting solution (Wright and Kinnicott)

No. 1.	Brilliant cresyl blue.....	0.1 gm.
	Distilled water	300.0 c.c.
No. 2.	Potassium cyanid	1.0 gm.
	Distilled water	1400.0 c.c.

No. 1 solution keeps permanently, but to prevent the growth of molds it should be kept in a refrigerator.

No. 2 solution does not keep and should be made up fresh every week or ten days. Immediately before use, mix *two* parts of number 1 with *three* parts of number 2, and filter.

Diluting solution (Reese and Ecker)

Sodium citrate 3.8 per cent aqueous sol....	100.0 c.c.
Formaldehyd 40 per cent.....	0.2 c.c.
Brilliant cresyl blue.....	0.1 gm.

Methods for Preparing Blood Smears

Slide Method

1. Clean slides with alcohol and polish with clean dry cloth. Pass through flame of Bunsen burner several times. Bon Ami may be used in place of alcohol.

2. Touch end of slide to drop of blood, which is procured in same manner as for cell counts.

3. Bring the slide in contact with a second slide, holding the one with the blood at an angle of 45°. As soon as blood spreads across the line of contact push the slide with an even sweep to the other end of second slide. In this manner the blood is drawn, not pushed across the slide. If the angle of the spreading slide is less than 45° the film will be thinner, on the other hand if it is more than 45° it will be thicker.

4. Dry film immediately by waving through the air or by blowing on it.

Cover Glass Method

1. On a perfectly clean cover glass take up a small drop of blood without touching the surface of the skin.
2. Drop the cover glass immediately on another cover glass so that the corners do not overlap.
3. As soon as the blood spreads out between the glasses draw them apart in a plane parallel to their surface.
4. Dry in the air.
5. Stain and mount on slides with balsam.

Methods for Staining Blood Smears

Hematoxylin and Eosin Method

1. Fix the smear by placing in methyl alcohol for 5 minutes.
2. Dry in the air.
3. Stain with Delafield's hematoxylin for 3 minutes, wash with tap water.
4. Stain with 1 per cent eosin for 1 minute; wash with tap water.
5. Dry thoroughly (gentle warming may be used) and examine; if cover glasses are used, mount on slides with balsam.

Wright's Method

1. Cover smear with 8 drops of Wright's stain and allow to remain for 2 minutes.
2. Add to the stain on the smear an equal amount of distilled water and allow the stain thus diluted to stand two minutes more (the stain should now have a "brassy" sheen).
3. Wash off with distilled water.
4. Dry and mount in balsam if cover glass is used.

Jenner's Method

1. Cover smear with Jenner's stain for 3 to 5 minutes.
2. Wash carefully with water until lavender color.
3. Dry, and mount in balsam if cover glass is used.

Differential Leukocyte Count

1. Examine blood smear after staining with Wright's or other suitable stain.
2. Note down in a column the names of the six varieties of leukocytes found in normal blood, namely, polymorphonuclear

neutrophiles; small lymphocytes; large lymphocytes; transitionals; eosinophiles and mast cells (basophiles).

3. Move slide systematically so as to avoid examination of same area twice. Record each leukocyte observed after its name (unrecognizable cells are to be omitted).

4. Count a total of one or more hundred and calculate the percentage of each.

5. Normal percentage of leukocytes in adult blood:

Polymorphonuclears	65 to 70 per cent
Small lymphocytes	22 to 36 per cent
Large lymphocytes	1 to 2 per cent
Transitionals	2 to 4 per cent
Eosinophiles	1 to 2 per cent
Mast cells (basophiles)	0.25 to 0.5 per cent

Examination of Fresh Wet Blood for Malaria

1. Use perfectly clean and grease-free cover glasses or slides.

2. Take up a small drop of blood by touching same with center of cover glass or slide.

3. Place on slide so that the blood will spread out in a thin film.

4. If examination is prolonged seal edges of cover glass with melted vaselin to prevent drying.

5. Examine at once with 1/12 oil immersion objective.

Examination of Stained Blood for Malaria

Thin Smear Method

1. Smears prepared as for differential leukocyte count but so thin that the red cells lie flat and well separated.

2. Fix and stain with Wright's or other suitable polychrome stain.

3. Dry and examine with 1/12 oil immersion objective.

Thick Smear Method

1. Place four or five drops of blood close together on a slide and immediately spread over surface about one inch square.

2. Place in the following solution until the hemoglobin has dissolved; 5 per cent solution of formalin containing 1 to 1.5 per cent of glacial acetic acid.

3. Wash with water.
4. Stain with Wright's or other suitable polychrome stain.
5. Dry and examine with 1/12 oil immersion objective.

Examination of Blood for Filaria

Wet Method

1. Puncture finger or lobe of ear and place large drop of blood on slide.

2. Immediately cover with cover glass and examine with low power lens.

3. The larvæ can be located by the disturbance they produce among the corpuscles.

The larvæ (Filaria bancrofti) are slender, about as wide as a red cell and about 0.2 to 0.4 mm. long. Some species appear periodically in the peripheral circulation and, in the case of Filaria bancrofti, they are most numerous at night. It is best to examine for this parasite at about 2 A. M. Should the above examination fail to reveal larvæ, the following method is recommended:

Concentration Method

1. Collect 1 c.c. of blood from ear or finger puncture in 5 c.c. of 2 per cent acetic acid.

2. Mix well and centrifuge.

3. Spread sediment on slide, cover with cover glass and examine with low power lens.

Staining Method

1. Make blood smears in usual manner or from sediment obtained by the above concentration method.

2. Dry, fix and stain by one of the methods for staining blood smears (see page 19).

Examination of Blood for Abnormal Leukocytes

1. Smears should be made thin so that the leukocytes lie flat and separated from other cells.
2. Stain with Wright's or other suitable stain.
3. Examine for the following:

   (*a*) Myelocytes (three varieties, namely, neuthrophilic, eosinophilic and basophilic); promyelocytes; metamyelocytes.

(*b*) Large and medium lymphocytes of acute lymphatic leukemia.

(*c*) Large numbers of small lymphocytes are found in chronic lymphatic leukemia.

Peroxidase Reaction

1. Make fresh thin blood smears.
2. Cover smear with Goodpasture's stain. Allow to stand one minute.
3. Add an equal quantity of water and allow to stand for three minutes.
4. Wash and dry.

This method of staining is used to differentiate leukocytes of marrow origin from lymphatic origin.

Polymorphonuclear neutrophile...granules stain intense blue
Polymorphonuclear eosinophile...granules stain intense red
Polymorphonuclear basophile.....granules are not stained
Myelocyte and myeloblast.....show deep blue granules
Lymphocytes .....cells stain a light red
Large mononuclear .....show a few blue-black granules
Transitional .....show a few blue-black granules
Erythrocytes .....buff color

Goodpasture's Stain

Sodium nitroprussid	0.05 gm.
Benzidine (C.P.) (Harmer)	0.05 gm.
Basic fuchsin	0.05 gm.
Hydrogen peroxid	0.50 c.c
Alcohol 95 per cent.....	100.00 c.c.

Dissolve the nitroprussid in a little water (1 or 2 c.c.) and add it to the alcohol, then add the other substances.

Iodophilia

1. Make fresh smear on cover glass.
2. Dry, but do not fix.
3. Place a small drop of iodin solution on slide and mount the cover glass in this solution.
4. Small brown masses in the polymorphonuclears will be seen in cases of iodophilia.

Iodin solution

Iodin	1 part
Potassium iodid	3 parts
Gum arabic	50 parts
Water	100 parts

The reaction is probably due to the presence of glycogen in these leukocytes.

Examination of Blood for Abnormal Erythrocytes

1. Smears should be thin so that the erythrocytes lie flat and separated from other cells.
2. Stain with Wright's or other suitable stain.
3. Examine for the following:

   (*a*) Abnormal changes in shape (poikilocytosis).
   (*b*) Abnormal changes in size (anisocytosis). If cell is too small it is called a microcyte, if too large it is called a macrocyte.
   (*c*) Changes in the staining property of the cell (polychromatophilia).
   (*d*) Granules in the cells taking the basic stain (basic granulation).
   (*e*) Nucleated cells as normoblasts, microblasts and macroblasts or megaloblasts.
   (*f*) Reticulated cells.

Method of Counting Reticulated Erythrocytes

1. Draw blood up to the mark 0.5 in the blood pipet marked 11.
2. Fill pipet to the mark 11 with diluting fluid.
3. Mix well by rotating pipet and mount on slide by placing a small drop on the slide and covering with cover glass.
4. Examine at least 500 erythrocytes and determine the percentage of these which are reticulated.

Diluting fluid

Potassium oxalate (2 per cent)	2 drops
Sat. solution of aqueous cresyl blue	1 drop
Normal salt solution	5 c.c.

Method of Vital Staining of Blood

1. Puncture finger and draw large drop into blood counting pipet.
2. Fill pipet with diluent.
3. Mix and allow to stand for 10 minutes.
4. Blow contents into test tube and centrifugalize.
5. Draw off supernatant fluid.
6. Take up sediment consisting of cells in capillary pipet, and make smear on warm slide.
7. Allow to dry and examine with 1/12 oil immersion objective.

Diluting fluid

Sat. solution of brilliant cresyl blue in 0.85 per cent NaCl solution ..... 5 c.c.
Salt solution, 0.85 per cent..... 5 c.c.
Sodium oxalate solution, 2 per cent..... 2 c.c.

Mix the oxalate and salt solutions first; then add the staining solution and filter.

Method of Determining the Tonicity or Resistance of Erythrocytes

1. Place twelve test tubes in a rack and mark them from 25 to 14.
2. Into each tube place as many drops of accurately prepared 0.5 per cent salt solution as the number indicated on the tube.
3. Add sufficient number of drops of distilled water to each tube to make the total volume 25 drops.
4. To each tube add 1 drop of whole blood to be tested. Best to test the blood immediately after drawing from vein.
5. Allow the tubes to stand at room temperature for one or two hours.
6. Read result. Reading should be made from tube marked 25 to tube marked 14, noting number on tube in which hemolysis begins and the first tube showing complete hemolysis.
7. Multiply the number on the tube by 0.02 to obtain the percentage of NaCl it contains.

Normal blood begins to hemolyze in 0.42 to 0.38 per cent NaCl and is completely hemolyzed in 0.36 to 0.32 per cent.

It is well to test a normal blood each time as a control.

Method of Determining the Coagulation Time of the Blood

Bogg's Method

1. The finger or ear is washed with alcohol and allowed to dry; puncture and wipe away first drop of blood.

2. Note the time on watch when second drop appears; place drop on cone of coagulometer and adjust in moist chamber.

3. Place chamber on microscope stage and examine with low power objective.

4. Squeeze rubber bulb (not too forcibly) about once every half to one minute, noting effect of air on corpuscles.

5. When the movement of individual corpuscles ceases and the drop moves as a whole or the corpuscles move toward the center and spring back to the periphery, note the time. The interval represents the coagulation time.

6. Duplicate readings should be made after the cone has been made perfectly clean and dry.

Normally the blood coagulates in 2 to 8 minutes.

Method of Determining the Bleeding Time of Blood

1. Puncture the lobe of the ear or the finger so that the blood flows drop by drop without any assistance.

2. Note the time the first drop appears.

3. Remove each drop as it forms with filter paper, care being taken not to touch the skin.

4. Note the time bleeding stops.

The time interval between the appearance of the first drop and the removal of the last represents the bleeding time. Normally it is 2 to 3 minutes.

Method of Estimating Bilirubinemia (Van den Bergh's)

1. Secure about 10 c.c. of blood by venipuncture and allow the serum to separate.

2. In a conical centrifuge tube place 2 c.c. of serum and 4 c.c. of 97 per cent alcohol. Mix with a fine glass rod.

3. Centrifuge and transfer 4 c.c. of the clear supernatant fluid to a 25 c.c. glass stoppered cylinder; add 1 c.c. of the diazo reagent, 2 c.c. of 97 per cent alcohol and mix.

4. After standing 15 minutes compare with the standard solu-

tion using a colorimeter which permits of very little evaporation, as that of Meulengracht. The standard should be of such strength as nearly corresponds to the color produced in the serum.

Calculations are as follows: Since the serum is diluted 5 times, multiply the colorimeter reading by 5; if, for example, the serum has a color corresponding to that of 5 parts per million standard solution, the bilirubin content of the blood would be equal to 25 parts per million.

Diazo Reagent.—Mix 0.75 c.c of solution No. 1, which is a 0.5 per cent solution of sodium nitrite in water, with 25 c.c. of solution No. 2, prepared by dissolving 1 gm. of sulphanilic acid in 25 c.c. of concentrated hydrochloric acid and diluting with water to one liter.

Bilirubin Standard Solution.—Dissolve 0.1508 gm. ferrous ammonium sulphate in 50 c.c. of concentrated hydrochloric acid and dilute to exactly 100 c.c. This stock solution keeps indefinitely. To 10 c.c. of this stock solution add 25 c.c. of concentrated hydrochloric acid and dilute to 250 c.c. This solution is used for comparison and keeps about six months. To 3 c.c. of this solution in a 25 c.c. mixing cylinder, add 3 c.c. of a 20 per cent solution of potassium thiocyanate and 12 c.c. of ether. Shake vigorously until the color is absorbed by the ether, which has been found to be equivalent to one part of bilirubin in 200,000 or 5 parts per million. It is well to check this color against a known solution of bilirubin dissolved in 97 per cent alcohol containing a few drops of 10 per cent sodium hydroxid and treated with the diazo reagent.

EXAMINATION OF THE URINE

The Reaction

Qualitative Test

Place a small piece of litmus paper in the urine; if the reaction is acid the paper will turn red, if neutral (amphoteric) no change will take place, if alkaline it will turn blue.

Quantitative Test (Folin's Method)

1. To 25 c.c. of urine add 1 and no more than 2 c.c. of a 0.5 per cent alcoholic solution of phenolphthalein, and 15 to 20 gm. of potassium oxalate.

2. Shake for about one minute and titrate at once, with n/10 sodium hydroxide, until the first trace of pink appears.

3. The result is expressed in terms of n/10 NaOH solution required to neutralize the total amount of urine for 24 hours or a shorter standard period.

The Specific Gravity

1. Fill cylinder or small graduate with urine.
2. Float hydrometer freely so it does not touch sides.
3. Read the number at the bottom of the meniscus.

If insufficient urine is received to float the hydrometer dilute the urine with an equal volume of distilled water. The last two numbers of the reading will have to be multiplied by two.

Qualitative Tests for Albumin

Heat and Acid Test

1. Filter urine if necessary to obtain perfectly clear specimen.
2. Fill two test tubes about one-third full.
3. Boil one tube, shaking gently to keep tube moist, and to prevent cracking of the tube.
4. Add two or three drops of acetic acid (never add the acid before boiling).
5. Compare this tube with the unboiled control against a black background.

If albumin is present in small quantity, a faint turbidity will occur in the boiled tube; if in larger quantity a marked turbidity or flocculent precipitate will occur. Phosphates may cause a turbidity upon boiling but this disappears upon the addition of the acid.

Exton's Test

1. Filter urine until clear.
2. Place a small amount in a clean test tube.
3. Add an equal quantity of the reagent.
4. Heat gently (do not boil).
5. If albumin is present the mixture becomes cloudy, which may be slight, or there may be a heavy precipitate depending upon the amount of albumin present.

Reagent

Sodium sulphate	200 gm.
Sulphosalicylic acid	50 gm.
Water to make	1000 c.c.

Dissolve the sulphate in 700 c.c. of water with heat; allow to cool, then dissolve the acid without heat and dilute to 1000 c.c.

Heller's Nitric Acid Ring Test

1. Filter urine if necessary until perfectly clear.
2. Place small amount of nitric acid in a clean test tube.
3. By means of a pipet and tilting, carefully overlay with urine.
4. Albumin forms a whitish ring at the line of contact between the urine and acid.

Robert's Ring Test

1. Filter urine, if necessary, until perfectly clear.
2. Place a small amount of reagent in the bottom of a clean test tube.
3. Overlay with urine.
4. A whitish ring at point of contact indicates presence of albumin.

Reagent

Saturated solution of magnesium sulphate	5 parts
Nitric acid	1 part

Spiegler's Test

1. Strongly acidify urine with acetic acid and filter.
2. Place a small quantity of reagent in a conical glass and carefully overlay with an equal amount of urine.
3. A white ring at the line of contact develops if albumin is present.

This test is too delicate for routine use as it may show a well marked ring when there is no abnormal albumin in the urine, but only a slight increase in the amount of nucleo albumin.

It may be used in cases where the detection of a trace is important. A negative reaction is of great value in showing the absence of albumin.

Reagent

Mercuric chlorid	8 gm.
Tartaric acid	4 gm.
Glycerin	20 c.c.
Distilled water	180 c.c.

Quantitative Tests for Albumin

Esbach's Method

1. Filter urine. If specific gravity exceeds 1.010, or if the presence of a large quantity of albumin is indicated by previous qualitative tests, dilute the urine with equal quantity of water.
2. Fill Esbach tube to the mark U with urine.
3. Add Esbach solution to the mark R.
4. Mix thoroughly.
5. Set aside for 24 hours at room temperature, or best at a temperature of 15 degrees C.
6. Note the level of the precipitate and calculate the number of grams per liter or the percentage.

The marks on the tube refer to grams per liter or tenths of 1 per cent.

Esbach Solution

Picric acid	10 gm.
Citric acid	20 gm.
Distilled water	1000 c.c.

Tsuchiya's Method

1. If the urine is alkaline acidify with a few drops of acetic acid.
2. Proceed in the same manner as in the Esbach method, using Tsuchiya's reagent in place of Esbach's.

Reagent

Phosphotungstic acid	1.5 gm.
Hydrochloric acid, conc.	5.0 c.c.
Alcohol (96 per cent)	95.0 c.c.

Test for Bence-Jones Protein

1. Filter urine; acidify with acetic acid if alkaline.
2. Heat slowly.
3. Bence-Jones protein will be precipitated on heating but on

subsequent boiling will partially or entirely disappear and reappear on cooling.

Qualitative Tests for Sugar

Benedict's Test

1. Place 5 c.c. of Benedict's qualitative solution in a clean test tube.
2. Add 8 to 10 drops of urine and mix thoroughly.
3. Boil thoroughly for several minutes; or tubes may be placed in a boiling water bath.
4. Allow to cool spontaneously.

If sugar is present a green, red, or yellow precipitate will form, the color depending upon the amount of sugar present.

If no sugar is present the solution will remain clear or show only a slight turbidity of a faint bluish color.

Reagent

Copper sulphate	17.3 gm.
Sodium citrate	173.0 gm.
Sodium carbonate (anhydrous)	100.0 gm.
Distilled water to make	1000.0 c.c.

Dissolve the citrate and carbonate in about 500 c.c. of distilled water by boiling. Filter through paper.

Dissolve the copper sulphate in about 100 c.c. of water.

Add the copper solution slowly to the citrate and carbonate solution and stir continuously while adding.

Measure and add sufficient water to make the total volume 1000 c.c.

Fehling's Test

1. Prepare Fehling's qualitative reagent by mixing equal parts of solutions No. 1 and No. 2; dilute with approximately four parts of water.
2. Place about 5 c.c. of the diluted reagent in a clean test tube.
3. Heat to the boiling point.
4. Add urine drop by drop until 5 c.c. have been added. Keep the solution at the boiling point until reaction takes place or 5 c.c. have been added.
5. If sugar is present a yellow or red precipitate will form.

If no sugar is present the solution will remain clear or a greenish precipitate may form.

Reagent

Fehling's Solution No. 1.	
Copper sulphate	34.65 gm.
Distilled water	500.00 c.c.
Fehling's Solution No. 2.	
Rochelle Salts	173.0 gm.
Sodium hydrate	125.0 gm.
Distilled water	500.0 c.c.

Keep solutions in separate bottles and mix equal quantity of each as needed.

Fermentation Test

1. Place 10 c.c. of urine in a test tube and add a piece of fresh Fleischman yeast about the size of a pea; mix gently to emulsify the yeast.

2. Transfer to a fermentation tube; make sure the arm is free of bubbles of air.

3. Place in an incubator for a few hours.

4. A normal urine and a normal urine to which is added a pinch of glucose may be treated in the same manner as negative and positive controls respectively.

5. A positive reaction is indicated by the collection of carbon dioxid gas in the arm.

6. If necessary, guard against gas production by bacterial fermentation by adding a pinch of tartaric acid (advisable if mixtures are incubated more than four hours).

Quantitative Tests for Sugar

Benedict's Method

1. Dilute 10 c.c. of urine with 90 c.c. of water.

2. Mix and place in a buret.

3. Place 25 c.c. of Benedict's reagent in a porcelain evaporating dish.

4. Add to the reagent 10 gm. of sodium carbonate and a little pumice, talc or a few glass beads.

5. Apply heat to reagent until boiling.

6. While the reagent is boiling run in the urine from the buret until the last trace of blue disappears. Keep the reagent boiling vigorously while the urine is being added.

7. Note the amount of diluted urine used.

Calculation.—25 c.c. of the reagent is reduced by 0.050 gm. of glucose. The amount of urine required to reduce this amount of reagent contains 0.050 gm. of sugar. The urine was diluted ten times; therefore one-tenth of the diluted urine represents the amount of undiluted urine containing 0.050 gm. of sugar. Divide 0.05 by the number of c.c. of undiluted urine to obtain the amount of sugar contained in one cubic centimeter of urine, then multiply this number by 100 to obtain percentage, or by the total number of c.c. in the twenty-four hour specimen to obtain the number of grams voided.

A short method to obtain the percentage is to divide 50 by the number of c.c. of diluted urine required to reduce 25 c.c. of the reagent.

Benedict's Test Tube Method

1. Place 5 c.c. of Benedict's reagent in a clean test tube.
2. Add 2 gm. of anhydrous sodium carbonate and a little pumice.
3. Heat to boiling over Bunsen burner.
4. While boiling run into tube from a 1 c.c. pipet, graduated in 0.1 c.c., undiluted urine until the last trace of blue has disappeared. The urine should be run in slowly and the solution kept boiling while the urine is being added.

If a very high percentage of sugar is suspected the urine can be diluted accordingly.

Calculation.—Divide the amount of undiluted urine into 1 to obtain the percentage of sugar.

Fehling's Method

1. If albumin is present, boil urine, add a few drops of acetic acid and filter.
2. Dilute 1 c.c. of filtered urine with 9 c.c. of water to make a 1 to 10 dilution.
3. Place in a test tube or small beaker 5 c.c. of Fehling's solution No. 1 and 5 c.c. of Fehling's solution No. 2, and dilute with 10 to 20 c.c. of water.
4. Boil to determine whether the copper solution is already reduced; this will be shown by the formation of a yellow or red precipitate. If reduced the solution should be discarded.
5. Add the diluted urine to hot Fehling's solution drop by drop from a buret or pipet graduated in 0.1 c.c. keeping the

Fehling's solution near the boiling point. Continue the addition of urine until the blue cupric salt has been entirely replaced by the yellow or red cuprous salt. The absence of any blue color is best determined after allowing precipitate to settle.

6. To determine if reduction is complete test as follows: With a clean glass rod transfer a drop to a hollow porcelain plate; wipe the rod and transfer a drop of acetic acid; wipe and transfer a drop of 10 per cent potassium ferrocyanid. Mix; if a brownish color develops reduction is not complete. If no color is produced reduction is complete. This method is to be preferred for determining the point of complete reduction.

Calculation.—Essentially the same as in the Benedict test (see page 43). 10 c.c. of Fehling's solution is reduced by 0.050 gm. of glucose. The amount of undiluted urine required to reduce this amount contains 0.050 gm. of sugar. The following formula can be used in this calculation as in the Benedict's: 50 divided by the amount of undiluted urine = the percentage of sugar.

Tests for Acetone

Rothera's Test

1. To 5 or 10 c.c. of urine add about 1 gm. of ammonium sulphate.
2. Add 2 or 3 drops of a freshly prepared 5 per cent solution of sodium nitroprussid.
3. Mix thoroughly.
4. Stratify with strong ammonium hydroxid.
5. If acetone is present a permanganate color will develop at the line of contact.

This test is very delicate for both acetone and diacetic acid.

Rothera's Test (Modified)

1. Place 3 to 5 c.c. of urine in test tube.
2. Add 1 c.c. of reagent and mix thoroughly.
3. Overlay with strong ammonium water.
4. A purple or permanganate ring develops at the junction of the fluids if acetone or diacetic acid is present.

Reagent

Ammonium nitrate	30 gm.
Sodium nitroprussid	2 gm.
Distilled water	80 c.c.

Test for Diacetic Acid

Gerhardt's Test

1. To about half a test tube full of fresh urine add a few drops of a 10 per cent ferric chlorid solution.
2. Filter off precipitated phosphates.
3. To filtrate add more of the ferric chlorid solution.
4. If diacetic acid is present the solution will turn a Bordeaux red color. In as much as this same color may be produced in the presence of certain drugs and substances other than diacetic acid it is necessary to repeat the test if positive with another sample which has been boiled for 10 or 15 minutes. If the reaction is due to diacetic acid negative results will be obtained with the boiled sample. If due to other substances positive results will be obtained with both the unheated and boiled samples.

Test for Beta-Hydroxybutyric Acid

Hart's Test

1. Dilute 20 c.c. of urine with an equal amount of water.
2. Reduce to one half its volume by boiling.
3. Dilute to 20 c.c. with water and place 10 c.c. in each of two test tubes.
4. To one tube add 1 c.c. of hydrogen peroxid and warm gently for one minute. Then allow to cool.
5. To both tubes add 10 drops of glacial acetic acid and 5 or 10 drops of freshly prepared sodium nitroprussid solution.
6. Mix thoroughly.
7. Overlay with strong ammonium water.
8. Allow to stand three or four hours.

A positive result is a purple ring in the tube treated with the peroxid and none in the other.

Estimation of Urea

Marshall's Urease Test

1. Soy beans are ground to a fine powder and preserved at room temperature in a well stoppered bottle.
2. Mix 2 gm. of bean powder with 20 c.c. of distilled water and allow to stand with occasional shaking for an hour.
3. Add 2 c.c. of n/10 HCl and place the mixture in a water

bath at 35° C. for a few minutes. Filter. To the filtrate add a few drops of toluene and keep in a well stoppered bottle. This solution will remain active for about 5 days at room temperature.

Tablets of urease are prepared by Hynson, Westcott and Dunning, Baltimore, which may be used in place of the above solution.

4. Place 1 c.c. of urine in each of two flasks of 200 or 300 c.c. capacity and add distilled water to 100 c.c. To one flask add 2 c.c. of the above solution or 2 tablets.

5. Heat both flasks to 40° C. for fifteen minutes.

6. To each flask add 5 drops of a 1 per cent alcoholic solution of methyl orange.

7. Titrate the contents of each flask to a distinct pink with n/50 HCl.

Calculation.—From the number of c.c. of n/50 HCl used to neutralize the specimen containing urease subtract the number required to neutralize the control. Multiply the result by the factor 0.6; this will give the number of grams of urea per 1000 c.c. of urine. Multiply this result by the factor 2.14 to obtain the amount of urea nitrogen.

Estimation of Chlorids

Volhard's Quantitative Method

1. Place 5 c.c. of urine which has been freed of albumin in a porcelain dish or flask.

2. Dilute with 20 c.c. of distilled water.

3. Add 10 c.c. of standard silver solution (each c.c. precipitates 0.01 gm. of NaCl).

4. Add 2 c.c. of indicator solution (ferric ammonium sulphate).

5. Mix thoroughly and titrate with standard ammonium sulphocyanate solution (1 c.c. precipitated by 1 c.c. of standard silver nitrate solution) until the first trace of yellow appears.

Calculation.—Subtract the number of c.c. of sulphocyanate solution used in titration from 10 and multiply by 0.01 to obtain the number of grams in 5 c.c. of urine; or subtract the c.c. of sulphocyanate used from 10 and multiply by 0.002 to obtain the amount per c.c. This result can be multiplied by the number of c.c. in the specimen to obtain the total chlorids voided.

Normal findings are between 10 and 15 gm. in 24 hours.

Standard Silver Solution

29.59 gm. silver nitrate (exact measure) dissolved in 1000 c.c. of water. Each c.c. should precipitate 0.01 gm. NaCl and should be tested against a known NaCl solution before use.

Ammonium Sulphocyanate Solution

Dissolve 13 gm. of ammonium sulphocyanate in 800 c.c. of water. One c.c. of this solution should precipitate one c.c. of the standard silver solution. Before use determine if 10 c.c. precipitates exactly 10 c.c. of silver solution; if not dilute or make stronger as it may indicate.

Indicator Solution

Dissolve 100 gm. ferric ammonium sulphate in 100 c.c. of a 25 per cent nitric acid.

Qualitative Method

1. Remove albumin if present by boiling after adding a few drops of acetic acid and filtering.
2. Place about 10 c.c. of albumin free urine in a test tube and acidulate strongly with nitric acid.
3. Add 5 drops of a 10 per cent solution of silver nitrate.
4. Treat a normal urine in the same manner, for comparison.
5. If chlorids are present in normal amount a heavy precipitate appears which settles quickly. If the quantity is diminished the precipitate will be light and if increased it will be very heavy compared with control (normal sample).

The test is useful for determining roughly any increase or decrease in the quantity of chlorid present in urine.

Test for Indican

Obermayer's Test

1. Add to about 5 c.c. of urine in a test tube an equal volume of Obermayer's solution and mix thoroughly.
2. Heat until tube is warm.
3. Add 2 c.c. of chloroform and shake tube rather violently to bring the chloroform in intimate contact with all parts of the solution.
4. Allow chloroform to settle.

If indican is present the chloroform will be colored blue, which will range from a trace to a very deep blue, depending upon the

amount present. If iodin or its salts have been ingested the chloroform may show a reddish tinge.

Obermayer's Solution

Ferric chlorid	2 gm.
Conc. hydrochloric acid	1000 c.c.

Tests for Bile, Bile Acids and Bile Pigments

Rosenbach's Modification of Gmelin's Test for Bile

1. Filter 100 c.c. or more of a urine through a small filter paper.
2. Remove filter paper from funnel and allow it to partially dry.
3. Touch the paper with a drop of old nitric acid.

If bile is present a spreading ring of rainbow colors with green on the outside and most marked will form.

Huppert's Test for Bilirubin

1. To 10 or 15 c.c. of urine add a saturated solution of calcium chlorid.
2. Filter.
3. Discard filtrate, and after perforating the filter wash precipitate into beaker with a small amount of alcohol acidulated with sulphuric acid.
4. Boil the solution.

If bilirubin is present the solution will assume a bright emerald green color.

Oliver's Test for Bile Acids

1. Filter a small amount of urine (5 or 10 c.c.) until perfectly clear.
2. Acidify with acetic acid (not necessary if already acid).
3. Dilute with water until sp. gr. is less than 1.008.
4. Place 2 c.c. in test tube.
5. Add 5 c.c. of reagent.

A positive reaction is indicated by a milky turbidity.

Reagent

Peptone	8.33 gm.
Salicylic acid	1.12 gm.
Water containing 2 drops of acetic acid	1000.00 c.c.

Filter until transparent.

Test for Urobilin

1. To 10 c.c. of urine add a few drops of Lugol's solution.
2. Mix with 10 c.c. of a saturated alcoholic solution of zinc acetate.
3. Filter.
4. The filtrate will show a green fluorescence if urobilin is present.

Test for Urorosein

1. Place 5 c.c. of urine in a test tube.
2. Add 5 c.c. of chemically pure HCl and mix thoroughly.
3. Add a few drops of a 1 per cent solution of sodium nitrite.
4. If urorosein is present a characteristic rose red color will develop.

Test for Diazo Substances

Diazo Test

1. Place in a test tube 40 parts or 2 c.c. of a 5 per cent solution of HCl saturated with sulphanilic acid and 1 part or 1 drop of 0.5 per cent solution of sodium nitrite and mix thoroughly.
2. Add an equal volume of urine and again mix.
3. Overlay with ammonium hydroxid.

A positive reaction will be shown by the development of a distinct red color at the point of contact of the two solutions; this will be diffused throughout the fluid on agitation and must color the foam a true salmon pink. Positive reactions may be obtained with normal urine. It is frequently positive in febrile conditions and has little practical value.

Technic of Phenolsulphonephthalein Functional Kidney Test

1. Have patient urinate or collect by catheter at a prescribed time and label specimen No. 1 (may be used for general examination).
2. Then give patient 10 to 12 ounces of water.
3. Thirty minutes later inject 0.006 gm. of the dye in the gluteal muscles (solution in ampules prepared by Hynson, Westcott and Dunning, Baltimore). Note time of injection.
4. Have patient void or catherize one hour later (No. 2) and again two hours after injection (No. 3).
5. Determine amount of dye excreted with Nos. 2 and 3. To

each add, drop by drop, 25 per cent NaOH until the maximum color is obtained. Then dilute each to 1000 c.c. with water.

6. Compare in a Duboscq or other colorimeter with a standard consisting of 0.003 gm. of dye in 1000 c.c. The cylinder containing the standard may be conveniently placed at the 10 mm. mark.

The Dunning colorimeter may be used instead if the colors are accurate.

7. Normally 40 to 60 per cent of dye is eliminated during the first hour and 20 to 25 per cent during the second hour, or a total of 60 to 85 per cent for two hours.

Microscopic Examinations of Urine

1. Allow specimen to stand for 6 to 12 hours in a cool place in a conical glass or centrifuge specimen for several minutes.

2. Remove sediment from bottom of glass or centrifuge tube by means of a pipet and place on glass slide.

3. With light almost cut off, examine with 2/3 objective and for further and more detailed examination use 1/6 objective.

The following should be looked for and note approximate number and kind present:

Casts—hyaline, granular, waxy, etc.; approximate number per field (1/6 objective).

Epithelial cells—squamous, round, cuboidal, etc.

Cylindroids—number per field.

Erythrocytes.

Pus cells.

Leukocytes—give approximate number per field (1/6 objective).

Crystals—acid urine: calcium oxalate, uric acid, urates, etc.; alkaline urine: triple phosphates, amorphous phosphates, etc.

Amorphous material.

Bacteria; spermatozoa, etc.

EXAMINATION OF GASTRIC CONTENTS

Test Meal. Ewalds: 2 pieces (35 gm.) of toast and 8 ounces (250 c.c.) of tea.

Retention Meal. Given in place of the regular evening meal: 4 ounces each of boiled string beans and rice. In many instances

an ordinary mixed diet will serve the purpose. Remove residuum next morning before breakfast.

Macroscopic Examination

1. Record the amount in cubic centimeters.
2. Record the amount of food present. Gross food in a fasting residuum should be measured.
3. Note the color.
4. Mucus—differentiate between stomach mucus and nose and throat mucus. The latter is in mass and stringy, the former is in flakes or shreds. Note the amount present as slight, moderate, large or very large amount.
5. Bile—the color of the specimen is an index for the presence of bile; it may be yellow or green. It is occasionally present in normal fasting residue but more commonly in pathologic conditions as gall bladder disease or hyperchlorhydria.
6. Blood (gross blood)—note if bright red or coffee color. Not usual to get gross blood from passing tube but may happen. Note the amount as slight, moderate, etc.

When recording amounts as slight, moderate, etc., it is often necessary to abbreviate the records and, in such cases and in routine work, the amount can be recorded as +1, +2, +3, or +4.

Estimation of Total Acidity

1. Filter through paper or cotton if much food or mucus is present.
2. Place 1 c.c. in porcelain dish.
3. Add a few drops of 1 per cent phenolphthalein in alcohol as indicator.
4. Titrate with n/10 NaOH and report number of c.c. required to neutralize 100 c.c. of gastric juice. The normal reading is between 40 and 60.

Estimation of Free Hydrochloric Acid

Töpfer's Method

1. Place 1 c.c. gastric juice in a white porcelain dish.
2. Add 1 or 2 drops of a 0.5 per cent alcoholic solution of dimethyl-amino-azo-benzol as indicator. If free HCl is present the fluid will turn pink or deep red depending upon the amount.

3. Add slowly from a buret n/10 NaOH until the last trace of pink disappears.

4. Read off the number of c.c. n/10 NaOH used, multiply by 100 and the result will represent number of c.c. of n/10 NaOH to neutralize the free HCl in 100 c.c. of gastric juice. Normal readings are from 20 to 40.

To express the result in per cent of HCl multiply the number of c.c. of n/10 NaOH required to neutralize 100 c.c. of gastric juice by 0.00365 which represents the amount of HCl in grams neutralized by 1 c.c. of n/10 NaOH.

Fractional Gastric Analysis

1. Insert Rehfuss tube and remove 5 to 6 c.c. samples at intervals of exactly 15 minutes from the time the test meal is eaten until the stomach is empty.

2. Filter each specimen through cheese cloth and examine residues for mucus, blood and food rests.

3. Titrate each specimen for total acidity and for free hydrochloric acid as per methods above described. Each may be tested in addition for peptic activity and protein.

Fractional gastric analysis is now being considered unreliable by some because of inaccuracies involved since the position of the tube in the stomach alters the results; the acidity of different portions varies according to place in stomach from which removed since the acidity is not uniform in the whole stomach contents.

Töpfer's Method of Gastric Analysis for Total Acidity, Free and Combined Hydrochloric and Acid Salts

1. To 1 c.c. of fluid in a white porcelain dish add a few drops of phenolphthalein solution (1 per cent alcoholic) and titrate to determine the total acidity (see page 61); end point is a pink color which does not become deeper upon adding another drop of gastric juice.

2. To 1 c.c. of gastric juice in a second dish add a drop or two of dimethyl-amino-azo-benzol solution and titrate to determine the free HCl (see page 61); end point is discharge of pink to canary yellow.

3. Place 1 c.c. of unfiltered juice in a third dish and add a few

drops of 1 per cent watery solution of alizarin, which gives a yellow color to the fluid. Run in slowly from a buret n/10 NaOH until a distinct violet color is produced which is free of red, and the addition of another drop makes no further change.

4. Note the amount of n/10 NaOH used in each titration.

5. The results are expressed in number of c.c. of n/10 NaOH required to neutralize 100 c.c. of gastric juice. The number of c.c. required for neutralizing 1 c.c. is therefore multiplied by 100; or 5 or 10 c.c. of gastric juice may be used for each titration and the results multiplied by 20 or 10 to give equivalents for 100 c.c. of gastric juice.

6. Calculations are made as follows:

(a) Amount of n/10 NaOH required for phenolphthalein acidity multiplied by 100 = total acidity.

(b) Amount of n/10 NaOH required for dimethyl-amido-azobenzol acidity multiplied by 100 = free hydrochloric acid.

(c) Amount of n/10 NaOH required for alizarin acidity multiplied by 100 = free acidity due to free HCl and acid salts (organic or inorganic).

(d) From A subtract C = combined hydrochloric acid (combined acidity).

(e) From C subtract B = acid salts.

The total acidity (A) should equal B + D + E.

If only a few cubic centimeters of juice are available titrate with the dimethyl indicator for free HCl; then add phenolphthalein indicator to the same and titrate for total acidity. The two indicators do not interfere with each other.

Protein Estimation

Wolff-Junghan Test

1. Filter gastric juice until clear.

2. Arrange a series of six test tubes in a rack.

3. Place 9 c.c. of distilled water in the first tube and 5 c.c. in each of the remaining five.

4. To the first tube add 1 c.c. of filtered gastric juice and mix thoroughly.

5. Remove 5 c.c. from the first tube and place it in the second tube, mix thoroughly and remove 5 c.c. and place in the third

tube, and so on until the sixth tube from which 5 c.c. will be discarded.

6. The tubes will now contain the following dilutions of gastric juice: 1-10, 1-20, 1-40, 1-80, 1-160 and 1-320.

7. Overlay the contents of each tube with 1 c.c. of Wolff's reagent.

8. The appearance of a white ring at the point of contact of the fluids constitutes a positive reaction.

9. Note the highest dilution which gives a positive reaction.

Positive reactions are obtained normally in dilutions up to 1-40.

Wolff's Reagent

Phosphotungstic acid	0.3 gm.
Conc. hydrochloric acid	1.0 c.c.
Alcohol (96 per cent)	20.0 c.c.
Distilled water to make	200.0 c.c.

Methods for Detecting Lactic Acid

Kelling's Test

1. Fill a test tube full with water; add a drop or two of a 10 per cent aqueous solution of ferric chlorid and shake thoroughly. The solution should have a slight yellowish tint when compared with plain water.

2. Divide the solution in two tubes and to one add a few drops of filtered gastric juice.

3. The appearance of a canary yellow color in the tube to which gastric juice was added indicates a positive reaction. Compare both tubes so that any change in color can be observed.

Strauss's Test

1. Fill the Strauss separatory funnel to the mark 5 with filtered gastric juice.

2. Add ether up to the mark 25.

3. Shake thoroughly and allow fluids to separate.

4. By opening stop-cock allow fluid to run out down to the mark 5.

5. Add distilled water up to mark 25.

6. Add two drops of the official tincture of ferric chlorid diluted in the proportion of 1-10.

If lactic acid is present, on shaking, the water will assume a

green color, the intensity of which will be an index of the amount of lactic acid present.

Uffelmann's Test

1. Place 5 c.c. of a 1 per cent carbolic acid solution in a test tube.
2. Add a 10 per cent ferric chlorid solution drop by drop until the solution develops an amethyst blue color.
3. Add 2 or 3 drops of gastric juice.

If lactic acid is present the solution will develop a canary yellow color.

Determination of Enzymes

Qualitative Test for Pepsin

1. Place 25 c.c. of gastric juice in a flask, 70 to 100 c.c. capacity. If the specimen does not contain free hydrochloric acid then add a few drops of 10 per cent hydrochloric acid (pepsin acts only in an acid medium).
2. Place in the flask with the gastric juice a disk of coagulated egg-albumin.

If pepsin is present the disk will begin to swell in from one-half to one hour and dissolve in about three hours.

Egg-Albumin Disks

(*a*) Boil an egg until the albumin is distinctly coagulated.
(*b*) Cut the albumin into small cylinders about 5 mm. diameter.
(*c*) Section the cylinders into small disks about 1 mm. thick.
(*d*) The disks can be preserved in glycerin until needed.

Quantitative Test for Pepsin

1. Dilute fresh egg-albumin 13 times with 0.4 per cent HCl (1 part of egg-albumin to 12 parts of 0.4 per cent HCl). This solution will contain about 1 per cent dry protein.
2. Take two test tubes and place 10 c.c. of the above solution in each.
3. To one of the tubes add 5 c.c. of gastric juice.
4. To the other tube add 5 c.c. of distilled water.
5. Incubate both tubes at 37° C. for one hour.
6. Estimate the amount of albumin in each tube by Esbach's method (see Esbach's test on page 39).

7. The difference between the amounts found in the two tubes represents the amount of albumin digested.

8. Normally about 90 per cent is digested in one hour.

Lee's Test for Rennin

1. Place 5 or 10 c.c. of neutral or amphoteric milk in a test tube.

2. Add 3 to 5 drops of gastric juice.

3. Place in the incubator for 15 to 20 minutes.

If rennin is present coagulation will occur. In this test it is sometimes difficult to tell whether the rennin or the acid in the gastric juice caused the coagulation; however, rennin is practically always present where there is HCl in the stomach and the test is only of value in those cases in which there is no HCl, to determine the presence or absence of a true achylia.

Riegel's Method

1. Place 5 to 10 c.c. of neutral or amphoteric milk in a test tube.

2. Add 5 c.c. of neutralized gastric juice.

3. Place in the incubator for 15 minutes.

4. If rennin is present coagulation will occur.

Methods for Detecting Bile

See tests for bile, bile acids and bile pigments in section on the Examination of Urine (page 55).

Tests for Occult Blood in Feces, Gastric Juice, Etc.

Benzidin Test

1. Place a knife point full of benzidin in a test tube (use Merck's blood testing benzidin).

2. Dissolve in 2 c.c. glacial acetic acid. Warm the solution if necessary.

3. Add 2 c.c. of hydrogen peroxid (3 per cent).

4. Add 10 to 15 drops of the fluid to be tested.

5. A positive reaction is indicated by the appearance of a blue or green color.

Feces should first be diluted with a small quantity of water and boiled. A simpler method is to spread the solid feces on a clean

slide and pour the solution over it. The same color reaction will take place if occult blood is present.

Phenolphthalein Test

1. Place material to be tested in test tube. If fluid, place 10 to 15 drops in the tube; if feces, place a small portion in tube and add about 2 c.c. of water and mix well.
2. Add 1 c.c. of the reagent.
3. Add 1 or not more than 2 drops of a 10 per cent solution of hydrogen peroxid.
4. A positive reaction is indicated by the appearance of a bright red color.

Reagent

(*a*) Make a 20 per cent solution of sodium hydroxid.
(*b*) Add 2 gm. of phenolphthalein.
(*c*) Add excess of zinc dust (20 or more grams).
(*d*) Heat gradually until colorless or slightly yellowish.
(*e*) Pour off supernatant fluid into colored bottle.
(*f*) Cover with liquid paraffin (20 to 30 c.c.).

Aloin Test

1. Macerate a small portion of feces with a little water in a mortar.
2. Pour in test tube and add ether to extract the fat.
3. Invert tube several times rather vigorously and pour off supernatant ether; if the ether is at all discolored the operation should be repeated.
4. Add 1/3 volume of glacial acetic acid to convert hemoglobin into hematin and thoroughly mix.
5. Add ether to extract hematin; invert tube several times and set aside to settle.
6. Into another tube place about 5 c.c. of aloin solution (made by dissolving a knife-pointful of aloin in about 10 c.c. of 70 per cent alcohol), 5 c.c. of ethereal extract of hematin and 5 c.c. of ozonized turpentine; shake thoroughly and set aside.
7. In the presence of occult blood an intense red color will develop in the lower portion of the fluid in from 2 to 10 minutes.

Guaiac Test

1. Obtain ethereal extract just as described in the aloin test.
2. Pour into a test tube about 5 c.c. of the ethereal extract;

add a few granules of gum guaiac and a few cubic centimeters of hydrogen peroxid.

3. In the presence of occult blood a violet or blue color will develop in the resultant mixture.

Method of Microscopical Examination

Unstained Preparation

1. Place a small drop of gastric residuum on slide and cover with a cover glass.

2. Examine with 2/3 and 1/6 objectives for the following:

Epithelia—squamous, columnar, or cuboidal; single, small groups or sheets; bile stained or not bile stained.

Leukocytes—number per field with 1/6 objective (normally 1 or 2).

Mucus—small coils, sheets or strings; bile stained or not.

Erythrocytes—few, many, large number.

Tissue fragments—if sufficient size to keep for sections.

Bacteria—note whether they occur in association with nasal or mouth mucus or whether they are intimately in contact with known stomach or duodeno-gall tract cells with or without bile staining.

3. Stain for Fat.—Mix a drop of fluid with a drop of Sudan III on a slide and cover with cover glass. Neutral fat globules will be yellow or red.

4. Stain for Starch.—Mix a drop of fluid with a drop of Lugol's solution on a slide and cover with cover glass. Starch granules will be blue or blue black.

Method of Examination for Bacteria

1. Make this film on slide, fix with heat and stain with dilute carbolfuchsin; dry.

2. Examine with 1/12 oil immersion objective for Oppler-Boas bacillus (*Lactobacillus boas-oppleri*), Staphylococci, yeast, sarcina, etc.

3. Note the organisms present and whether in small or large numbers, single, in groups or colonies (masses).

EXAMINATION OF BILE AND DUODENAL FLUIDS

General Examination

1. Note the amount, reaction, viscosity, color, mucus, flocculi and test for occult blood (see page 71).

Microscopic Examination, for Bacteria, Pus and Cells

Unstained

Place one of the flakes obtained from the bile on a slide, cover with a cover glass and examine with low and high objectives for the following:

1. Epithelial cells—note their shape (oval, cuboidal, tall or short, columnar, etc.); arrangement (single clusters, rosettes, etc.) and whether or not they are bile stained.

(*a*) Oval or cuboidal or low columnar = duodenal mucosa cells (not bile stained).

(*b*) Columnar cells, bile stained = any part of gall tract.

(*c*) Short columnar cells bile stained = smaller gall ducts.

(*d*) Tall columnar cells bile stained = gall bladder or large gall ducts.

(*e*) Very deeply bile stained tall columnar cells = usually from gall bladder.

2. Leukocytes—number per field high power. Note if in clumps and whether or not they are bile stained.

3. Crystals—cholesterin, calcium, bile salts or pigments.

4. Bacteria—roughly estimate their number, whether or not they occur in clumps or colonies and whether they are associated with the bile stained elements or non-bile stained elements.

Stained Preparations

1. Select material and smear on slide in same manner as preparing sputum for examination for tubercle bacilli (avoid thick smears).

2. After staining with carbolfuchsin or Gram's method wash in water and examine with oil immersion lens.

3. Not usually as satisfactory as unstained specimens.

Determination of Trypsin, Amylase and Lipase

Einhorn's Method

1. Place sufficient bile in test tube to make it about 3 inches deep.

2. Place in the bile the following tubes (file off the ends, avoiding dried out ends):

(*a*) Hemoglobin tube.
(*b*) Starch tube.
(*c*) Olive oil tube.

3. Place in the incubator at 37° C. for 24 hours, and examine as follows:

4. Examine the two ends of the hemoglobin tube for clear digested area; measure it with millimeter rule, and the readings from both ends; take the average and report in numbers of millimeters. The digestion is due to the presence of trypsin.

Examination of starch tube—push the contents of tube out on a slide and cover with Lugol's solution, measure the part which does not turn blue as above and report in millimeters. The digested portion fails to give the starch reaction with iodin. The digestion is due to the presence of amylase.

Examine the ends of the olive oil tube and measure the portion which has turned violet. The Nile blue in the digested portion turns violet report in number of millimeters as above. The digestion is due to the presence of lipase.

Preparation of Starch Tubes

Agar powder	2.5 gm.
Starch	5.0 gm.
Distilled water	100.0 c.c.

Rub starch and agar in a mortar with sufficient water to make smooth paste, then add balance of the water.

Place in a flask and heat to boiling point.

Draw by suction while still hot into warm capillary glass tubing (inside diameter 1.5 mm.).

After cooling cut into small pieces (3 cm. lengths) and seal the ends with paraffin.

Preparation of Olive Oil Tubes

Olive oil	25 c.c.
Agar powder	2 gm.
Aq. sol. of Nile blue sulph. (1 to 2000 c.c.) q.s.	100 c.c.

Rub the olive oil and agar together to make thin paste and then add Nile blue solution to make 100 c.c. Heat to boiling; fill in capillary tubes as directed for starch tubes.

Preparation of Hemoglobin Tubes

Hemoglobin powder	1.0 gm.
Agar powder	2.5 gm.
Distilled water q.s.	100.0 c.c.

Rub hemoglobin powder with 10 c.c. of water until a smooth paste is obtained; then add the agar and water. Heat and fill capillary tubes as directed for starch tubes.

Phenoltetrachlorphthalein Liver Function Test

Method of Piersol and Bockus

1. Pass duodenal tube (any tube) into fasting stomach until tip is approximately 21 inches from lip.
2. Give patient two glasses of water by mouth and aspirate until clean; or give stomach lavage with small buret.
3. Introduce 60 c.c. of water into stomach to be retained.
4. Turn patient on right side.
5. Push tube slowly downward until tip is 27 inches from lips.
6. Make certain the tube is in the duodenum before proceeding further.

Indications are: duodenal tug; aspirating pure yellow alkaline bile; appearance of golden yellow froth; failure to obtain water upon aspirating. A good test is to introduce 30 c.c. of water by gravity and then lower tube and siphon back the water, which should be bile stained if tube is duodenum. Lyon's stethoscopic method may be used.

7. As soon as tube enters duodenum give patient 500 c.c. of water by mouth.
8. When a steady drip of bile-stained fluid comes from the tube inject intravenously 0.150 gm. of the disodium salt of phenoltetrachlorphthalein as prepared in ampules by Hynson,

Westcott and Dunning (each c.c. contains 0.050 gm. of the dye).

9. Record time of injection of dye (starting point).

10. Allow bile to drip into a white basin containing 3 or 4 c.c. of a 40 per cent solution of sodium hydroxid.

11. Record time of appearance of first pink color.

12. Record time of appearance of maximum color (deep purple).

13. Keep each half hour's output separate and mark them Nos. 1, 2, 3, and 4. Each basin used should not have more than 1 c.c. of a 40 per cent solution of sodium hydroxid in it (avoid excess).

14. Collection of fluid should continue over a period of two hours, taking the time of the injection of the dye as the starting point.

15. Estimate the amount of dye in each half hour sample.

Normal total output of dye—19 to 27 mgm. Average 22.5 mgm.
Normal appearance of first pink color—5 to 11 minutes.
Normal appearance of maximum color—8.75 to 15 minutes.
Normal elimination of dye for first hour—average 13.1 mgm.
Normal elimination of dye for second hour—average 8.9 mgm.

Method of Estimating Dye in Fluid

1. Each half hour sample examined separately. If sodium hydroxid was not added at time of collection a 40 per cent solution should be added drop by drop until maximum color is reached.

2. Dilute to 1000 c.c. with water.

3. Add 3 c.c. of a saturated aqueous solution of basic lead acetate and 1 c.c. of a saturated solution of calcium chlorid; heat until precipitate forms (usually a few minutes).

4. Filter; place about 10 c.c. of the filtrate in a test tube (same size as used in the standards) and make comparisons to determine the amount of dye present.

Preparation of Color Standards

1. To 193 c.c. of water add 6 c.c of a 10 per cent solution of sodium hydroxid.

2. Add exactly 0.1 c.c. of phenoltetrachlorphthalein solution (same as used in test; 50 mgm. per c.c.).

3. In a series of 10 test tubes place the following amounts of

the above solution (use tubes of uniform size): 0.2, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 4.0, 4.4, 4.8, 5.2, 5.6, 6.0, 8.0 and 10.0 c.c.

4. Add sufficient sodium hydroxid solution (10 per cent) to each tube to make the total volume 10 c.c.

5. The tubes thus prepared will represent the following: 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, and 25 mgm. of dye in 1000 c.c.

6. If properly sealed these standards will keep for from four to six weeks unchanged.

Rosenthal Method

1. Weigh the patient. The dose of phenoltetrachlorphthalein to be injected intravenously is 0.005 gm. per kilo (2.2 pounds). A 5 per cent solution may be employed, the dose for an adult of 132 pounds being 6 c.c.

2. Draw the proper dose of dye into a sterile 25 c.c. Record or Luer syringe and dilute with saline solution to the 25 c.c. mark.

3. Apply a tourniquet to the arm above the elbow and cleanse the skin with tincture of iodin and alcohol. Puncture a vein with a needle of moderate bore (gauge 16) *which fits the syringe* and allow 10 c.c. of blood to flow into a clean, dry test tube.

4. Attach the syringe and slowly inject the dye.

5. Fifteen minutes, 1 hour and 2 hours later remove 5 c.c. of blood from the opposite arm into clean, dry test tubes.

6. Allow the sera to separate from the clots and transfer each to separate small test tubes; centrifuge each if necessary to remove corpuscles.

7. To each tube add one drop of 5 per cent sodium hydroxid for every cubic centimeter of serum.

8. Compare the color of sera removed 15 minutes, 1 hour and 2 hours after injection with the serum secured before injection. Normally only a trace, 2 to 6 per cent, of the dye is present in the 15 minute specimen and practically none in the 1 and 2 hour specimens. In cases of dysfunction of the liver the dye may be retained for longer and variable periods in the blood.

9. The amount present may be determined colorimetrically as follows: Place 0.010 gm. of the dye (0.2 c.c. of a 5 per cent solution) in 100 c.c. of water (designated as 100 per cent standard solution). In each of a series of six small test tubes place 0.6 c.c.

of serum from the pre-injection sample of blood. To No. 1 add 0.2 c.c. of the 100 per cent standard solution; to No. 2, 0.2 c.c. of an 80 per cent standard solution; to No. 3, 0.2 c.c. of a 60 per cent; to No. 4, 0.2 c.c. of a 40 per cent; to No. 5, 0.2 c.c. of a 20 per cent, and to No. 6, 0.2 c.c. of a 12 per cent. The percentage of standard solutions will now be 25, 20, 15, 10, 5 and 3 per cent respectively. Compare the 15 minute, 1 hour and 2 hour sera with these in good light. The 100 per cent standard solution contains 0.010 gm. of dye to 100 c.c. of water. The percentages thus derived indicate approximately what part of the total amount of dye injected remains in the serum.

Widal and Abrami's Hemoclastic Crises Tests

1. Make a leukocyte count on fasting stomach (instruct patient not to eat before retiring and refrain from eating until count is made next morning).
2. Give patient 350 c.c. of milk by mouth.
3. Make a leukocyte count one hour after giving the milk.
4. Make a leukocyte count at the end of the second hour.
5. *Normally* in adults there should be a digestive leukocytosis. In liver dysfunction a leukopenia develops, i.e., the blood count at the end of one or two hours or both will be 200 to 5000 less than the original count on fasting stomach depending on severity of liver disease.
6. This test is only applicable to adults.

EXAMINATION OF FECES

Macroscopic Examination

1. Note general color, consistency, odor and the presence of blood, mucus, or the evidence of fermentation (gas). Note shape, if sausage the approximate diameter; ribbon shaped; small scybalous masses, etc.
2. Place a portion of the stool on gauze or wire sieve and directing a stream on the mass, macerate or break it up with a glass rod. Some idea of the general digestion may be thus obtained especially in regard to vegetable fiber. It also facilitates the detection of parasites and concretions.

Microscopic Examination

1. Macerate a small portion of feces in water and dilute to fill centrifuge tube. It is sometimes better to make an emulsion of stool on a slide, as centrifuging may cause the fat to come to the surface.

2. Centrifuge a few minutes; pour off supernatant fluid.

3. By means of a pipet take up a portion of the sediment and place three small drops on a slide, one at each end and one in the middle.

4. Mix one of the end drops with a little Lugol's solution; then mix the drop at the other end with a solution of sudan III.

5. Cover each drop with a cover glass.

6. Examine with the low and high power objectives the drop treated with Lugol's solution for the presence of starch (starch granules are stained blue with iodin).

7. Examine the drop at the opposite end for neutral fat (neutral fat stains red or yellow with sudan III).

8. Examine the middle drop which is unstained for fatty acid and other crystals; soaps; muscle fibers, mucus, pus, blood, cellulose, parasite eggs and larvæ.

9. Stain a smear by the method of Gram (see section on Bacteriology). Report approximate proportions of Gram + and — bacilli and cocci; report presence or absence of organisms that may be recognized morphologically, as Streptococci.

Method of Laboratory Diagnosis of Hookworm Disease

1. Dilute a small portion of feces with water. If stool is liquid this is not necessary.

2. Place drop on slide and cover with cover glass.

3. Examine with low power lens for ova (eggs).

The ova show a thin but distinct shell and a finely granular protoplasm which is segmented. There is a clear zone between the protoplasm and the shell. The adult worm seldom appears in the feces. The species common to Europe and Egypt is the *Ancylostoma duodenale* and that common to America, central and southern Africa, is the *Necator americanus*. The ova of both are very similar except in size, the latter being the largest. The *Ancylostoma duodenale* measures 32 to 38 by 52 to 61 microns

The *Necator americanus*, 36 to 45 by 64 to 76. The following method is recommended when the above examination is negative.

Pepper's Method

1. Sediment the feces by diluting with several volumes of water and allow to settle. The water is poured off and fresh replaced. This is repeated until no more matter floats. The final sediment is examined.
2. Place a drop of the final sediment on a slide and allow to stand for a few minutes.
3. Gently immerse in water. (The ova will cling to the glass and will not be washed away.)
4. Place another drop on the same slide and gently wash. Repeat until several drops have been washed.
5. After last drop has been washed, cover with cover glass and examine with low power for ova.

Cultivation of Hookworm Larvæ

1. Spread a small mass of feces on a thick pile of small filter papers.
2. Place in a Petri dish.
3. Pour water in the dish around the pile of paper (avoid submerging the paper).
4. Place in incubator for few days.
5. Remove the water and centrifuge it.
6. Examine the sediment for larvæ.
7. Larvæ which hatch out will find their way to the water.

Eosinophilia is a characteristic blood change and should be looked for in all cases of suspected hookworm disease.

Diagnosis of Other Forms of Intestinal Helminthiasis

1. An emulsion of feces may be examined by placing a drop on a slide, covering with a glass, and inspected with the 2/3 and 1/6 objective for the ova of *Ascaris lumbricoides, Tænia saginata, Bothriocephalus latus, Oxyuris vermicularis, Strongyloides intestinalis*, etc.
2. Sometimes better results are obtained by microscopical examination of sediment as described above for the ova of the hookworms.

Method of Laboratory Diagnosis of Trichinosis

Wet Blood Examination

The concentration method for the detection of Filaria with slight modification is recommended. It is necessary to collect 5 to 10 c.c. of blood from a vein in 25 to 50 c.c. of acetic acid solution and proceed as outlined under the above method (see page 23).

The larvæ are about 125 microns long by 6 microns broad. They only appear in the blood during their migration to the muscles, and may be detected between the sixth and twenty-second day after the onset of symptoms.

Examination of the Feces

During the diarrheal stage the stools should be examined for adult worms, particularly dead males or for actively motile embryos. These embryos measure about 90 by 6 microns.

Examination of the Blood

As eosinophilia is usually present, a differential leukocyte count should be made.

Method of Laboratory Examination for Intestinal Flagellates

1. Several methods may be employed as follows:

   (a) Dark field illuminator (see page 151).

   (b) India ink method (see page 145).

   (c) Smears may be made which should be fixed in methyl alcohol or 5 per cent formalin solution and stained by Giemsa's method.

   (d) Place a drop of Gram's iodin on slide. Mix portion to be examined. Cover with cover glass and examine. Flagellates are very active and are readily found with 1/6 objective.

Trichomonas intestinalis.—Pear shaped, three flagella anteriorly and a fourth projecting posteriorly. It measures about 9 by 15 microns. Found in the large intestines.

Chilomastix mesnili (Tetramitus mesnili).—This differs but little from the *Trichomonas intestinalis.* It has no fourth flagellum posteriorly, and the anterior flagella are longer.

Giardia intestinalis (Lamblia intestinalis).—Pear shaped with

a depression at the blunt anterior end. Three pairs of flagella around the depression and one pair project from the small tail-like projection. Size 10 by 15 microns.

Other flagellates as Cercomonas, Bodo, Prowazekia, etc., may be encountered and should be differentiated by the number of flagella, presence or absence of undulating membrane and blepharoplast.

Examination for Occult Blood

Same as described for occult blood in gastric contents (see page 71).

Examination for Bilirubin and Hydrobilirubin

1. If feces are hard and dry add a little water to reduce to a mushy consistency.
2. Emulsify a small portion in a saturated aqueous solution of mercuric chlorid and set aside for a few hours (this can be done on glass slides).
3. In the presence of hydrobilirubin, which is the normal pigment, the mixture will assume a faint pink color. If bilirubin is present a green color will be produced.

SPUTUM EXAMINATIONS

Macroscopic Examination

1. Pour a portion of the specimen in a Petri dish, spreading it out in thin layer over the bottom.
2. Note the color, odor, consistency and presence of blood.
3. Place the plate on black top of table or on black paper and examine carefully for the following:

(a) Curschmann's spirals—yellowish-white masses having a spiral twisted appearance, surrounded by a thinner homogeneous material.

(b) Diettrich's plugs—yellowish-white colored bodies varying in size from millet seed to bean, having a very disagreeable odor.

(c) Concretions—calcified material (gritty).

(d) Fibrinous casts.

Microscopic Examinations

Unstained

1. Select portions which appear from the macroscopic examination as being suspicious of any of the following: pus, Curschmann's spirals, elastic fibers, etc.

2. Place on slide, cover with cover glass and examine for the following:

(a) Heart failure cells—large endothelial cells four or five times the size of leukocytes and containing blood pigment (hemosiderin).

(b) Curschmann's spirals—skeins or twists or fine fibers through the center of which a light refractile line will be seen. Occasionally in or near the spirals will be found the diamond shaped Charcot-Leyden crystals.

(c) Elastic fibers—fibers having alveolar formation.

(d) Charcot-Leyden crystals — colorless and diamond shape.

Stained

1. Prepare thin smear of sputum on slide.
2. Dry in the air.
3. Fix by gentle heat over flame.
4. Stain with Wright's stain as described under blood (see page 19).
5. Examine with oil immersion lens.
6. Stain for tubercle bacilli, and by Gram's method, as described in the section on Bacteriology.

Examination for Albumin

1. Mix 10 c.c. of sputum with an equal quantity of water and 2 c.c. of a 3 per cent solution of acetic acid (to remove mucin).
2. Filter through paper.
3. Test filtrate for albumin using methods outlined under urine (see page 35).

Examination for Blood

1. Blood may be recognized by naked eye inspection; or by microscopic examination.

2. Occult blood tests may be conducted in same manner as described on page 71.

EXAMINATION OF CEREBROSPINAL FLUID

Macroscopic Examination

1. Color—normally like distilled water; may be clear but yellowish (xanthochromia).

2. For appearance—normally clear; may be opalescent, turbid or purulent.

3. Color of sediment—whether white, yellowish, grayish or greenish.

4. For coagula—normally none; may show flakes; cobweb coagula. May solidify (called massive coagulation and usually associated with xanthochromia, syndrome of Froin).

5. For blood—by naked eye appearance.

Total Cell Count

1. Should be made immediately or within a few hours after withdrawal of fluid by spinal puncture. Worthless if the fluid contains enough blood for naked eye detection. Counts are too low if made after coagula have formed.

2. Use white corpuscle pipet drawing diluting fluid to 1 and spinal fluid (shaken) to the mark 11.

3. Shake well; discard first drop or two and place drop in Fuchs-Rosenthal counting chamber. Allow cells to settle for 10 minutes.

4. Count all cells in ruled off area (erythrocytes are hemolyzed); multiply by 11 and divide by 32 = number of cells per cubic millimeter of undiluted fluid; or multiply cells counted by factor 0.35.

5. If a Fuchs-Rosenthal chamber is not available, use ordinary blood counting chamber (not as satisfactory). Count all cells in ruled off area (400 small squares) and multiply by 100 = number of cells in 1 cubic millimeter of undiluted fluid. Or the chamber may be filled with undiluted spinal fluid (if blood is not present and fluid is clear or not more than opalescent); count all cells in ruled off area (400 small squares) and multiply by 10.

6. Normally there are none to 10 cells per cubic millimeter; 10 to 12 borderline; more than 12 pathological.

Diluting fluid

Glacial acetic acid	0.5 c.c.
Distilled water	25.0 c.c.

Few drops of Loeffler's methylene-blue.

Differential Cell Count and Cytodiagnosis

1. Centrifuge fresh specimen of fluid.
2. Pour off supernatant fluid and make thin smear of sediment as described for blood.
3. Dry in air.
4. Stain with Wright's stain, or with methylene-blue.
5. Count and tabulate cells (lymphocytes, polymorphonuclears and endothelial cells) as described under method for differential count of leukocytes, and determine the number of each variety per 100 cells.
6. Normally, only lymphocytes and occasional endothelial cells are found.

Acute suppurative meningitis due to pneumococcus, meningococcus, Streptococcus, etc.—polymorphonuclear cells predominate in acute stage.

Tuberculous meningitis—small lymphocytes predominate.

Acute anterior poliomyelitis—polymorphonuclears early; later small lymphocytes (usually).

Syphilis (paresis, tabes, etc.)—small lymphocytes predominate.

Meningismus (serous meningitis; acute meningeal congestion)—endothelial cells predominate.

Qualitative Tests for Protein

Pandy Test

1. Place about 1 c.c. of a saturated solution of phenol in a test tube.
2. Add 1 drop of fluid to be tested.
3. If there is an increased amount of globulin present a bluish white ring or cloud is formed immediately.

Reagent

Place 100 c.c. of pure carbolic acid (melt crystals by standing bottle in hot water) in a bottle and add water up to 1000 c.c. Shake vigorously and stand in incubator for several days. Carefully pipet off supernatant fluid or use it direct from the bottle without disturbing the layer of acid.

Noguchi Test

1. In a small test tube place 0.2 c.c. of the fluid to be tested.
2. Add 1 c.c. of a 10 per cent solution of butyric acid in normal salt solution.
3. Heat to boiling.
4. Add 0.2 c.c. of normal sodium hydroxid.
5. Allow to stand for 5 to 10 minutes.
6. A faint opalescence is normal; a precipitate indicates a positive reaction.
7. Test objectionable on account of odor of butyric acid.

Ross-Jones Test

1. Place 1 c.c. of saturated ammonium sulphate in test tube.
2. Overlay with 1 c.c. of the fluid to be tested.
3. The appearance of a turbid ring at the point of contact indicates a positive reaction. Normally no ring at all is formed.

Ammonium sulphate reagent

Place 85 gm. of Merck's purified and neutral ammonium sulphate and 100 c.c. of distilled water in an Erlenmeyer flask; heat to boiling until all of the salt is dissolved. Cool slowly and filter.

Nonne-Apelt Test

1. *Phase I, for Globulin.*—In a small test tube place 2 c.c. of spinal fluid and 2 c.c. of the ammonium sulphate reagent (see above). Allow to stand 3 minutes. Compare with spinal fluid. A normal fluid gives no reaction or but a faint opalescence.

2. *Phase II, for Albumin.*—Filter contents of tube just prepared; acidulate with acetic acid and boil. A normal fluid remains clear or but faintly opalescent.

Quantitative Protein Tests

Mestrezat Test

1. Place 2 c.c. of spinal fluid in a test tube. Add 6 drops of 33 1/3 per cent solution of trichloracetic acid and heat to boil-

ing. A faint opalescence is normal. By centrifuging the precipitated protein may be roughly gauged.

2. In repeat tests use tubes of same size which permits gauging of protein and whether increasing or decreasing.

Esbach and Tsuchiya Tests

Conducted in same manner as described for urine on page 39.

Sugar Estimation

Qualitative

Normal cerebrospinal fluid contains dextrose which reduces Fehling's solution; in acute suppurative meningitis due to meningococci, Streptococci, pneumococci, etc., this power is less or entirely lost, due probably to consumption of the dextrose by the organisms.

1. Examine the fluid in a fresh state.

2. To 1 c.c. each of Fehling's alkaline and copper solutions add 10 c.c. distilled water; boil; if color remains unchanged it is fit for use.

3. In a small thin walled test tube place equal parts of cerebrospinal fluid and diluted Fehling's solution; heat to boiling and note presence or absence of reduction (deep violet color is due to protein).

Quantitative

1. The same method described for the estimation of blood sugar (see page 317) can be applied to cerebrospinal fluid with but slight modifications. The fluid should be diluted with four volumes of distilled water and used in the place of blood filtrate. Changes in the calculation will be necessary.

Quantitative Estimation of Chlorids

The method described for the quantitative estimation of this substance in the blood may be applied to cerebrospinal fluid (see page 321).

Colloidal Gold Test

1. Place eleven chemically clean test tubes in a rack.

2. Into the first tube place 1.8 c.c. of 0.4 per cent NaCl solution and 1 c.c. in each of the remaining ten tubes.

3. Add 0.2 c.c. of spinal fluid to the first tube and thoroughly mix.

4. Remove 1.0 c.c. from the first tube and place in the second tube; mix thoroughly and remove 1.0 c.c. and place in the third tube; continue until the tenth tube is reached and then discard 1.0 c.c. from this tube. The eleventh tube is used as a control.

5. Add to each tube 5 c.c. of colloidal gold reagent.

6. Mix thoroughly and set aside for twenty-four hours, at the end of which time the readings are made.

7. Readings—each tube is examined and the reaction recorded using the numbers 0 to 5.

0 = unchanged as compared with the control
1 = reddish blue
2 = lilac or purple
3 = blue
4 = almost colorless (trace of blue)
5 = colorless

8. The readings are recorded in the order the tubes stand, *e.g.*, 5, 5, 5, 5, 4, 3, 1, 0, 0, 0, this represents a curve in the paretic zone; 1, 1, 2, 3, 2, 1, 0, 0, 0, 0, this represents a curve in the luetic zone; 0, 0, 1, 1, 1, 2, 2, 3, 2, 1, this represents a curve in the meningitic zone.

Preparation of Colloidal Gold Reagent (Miller, Hammers and Brush modified by Perlman)

Apparatus required:

Beakers, pyrex glass, 500 c.c. capacity.
Flasks, pyrex glass, 1 or 2 liter capacity for collecting water.
Pipets, five 5 c.c. graduated in 1/10ths.
Bottles, three, glass stoppered, 100 c.c. capacity.
Glass rod, 18 inches for stirring.

Cleaning glassware (important):

Wash well with soap and water, rinse with tap water by running large stream into beakers, flasks, graduates and bottles and forcing water through pipets. Place a small amount of HCl in each and by rotating bring it in contact with all parts of the interior. Rinse well with tap water and leave filled with water until just before use, then rinse with distilled and lastly with double distilled water.

Reagents: Double distilled water (the last distillation should be done the same day it is to be used).

Gold solution 1 per cent:

Gold chlorid (Merk's yellow crystals)	1 gm.
Double distilled water	100 c.c.

Potassium carbonate solution 2 per cent:

Potassium carbonate (Merk's blue label)	2 gm.
Double distilled water	100 c.c.

Oxalic acid solution 1 per cent:

Oxalic acid (Merk's blue label)	1 gm.
Double distilled water	100 c.c.

Technic of preparation:

Into a beaker cleaned as outlined above place 500 c.c. of double distilled water.

Add 5 c.c. of 1 per cent gold solution.

Place beaker on asbestos filled wire gauze and heat over Bunsen burner until boiling point is reached. Stir the solution to keep the heat distributed while heating.

As soon as the solution begins to boil, turn off the flame and add 5 c.c. of 2 per cent potassium carbonate solution and 5 c.c. of 1 per cent oxalic acid solution. These last solutions can be added rapidly and should color appear before all of the oxalic acid has been added continue to add the full 5 c.c.

The solution should develop a salmon or orange-red color and be perfectly clear; it should fullfil the following requirements:

(*a*) Neutral to alizarin (place 5 c.c. in a test tube and add 2 drops of a 1 per cent solution of alizarin red in 50 per cent alcohol).

(*b*) Transparent and salmon or orange-red color.

(*c*) 5 c.c. should be completely precipitated in one hour by 1.7 c.c. of 1 per cent NaCl solution.

(*d*) It should give a typical paretic curve with a known paretic fluid.

(*e*) No more than a No. 1 reaction should be obtained with known normal spinal fluid.

Colloidal Mastic Test

1. Arrange six small test tubes in rack.

2. Place 1.5 c.c. of alkaline-saline solution in the first tube and 1 c.c. in each of the remaining five.

3. Add 0.5 c.c. of spinal fluid to the first tube, mix thoroughly and transfer 1 c.c. to the second tube.

4. Transfer 1 c.c. from the second tube to the third and so on until the fifth tube from which 1 c.c. is discarded. The sixth tube is used as a control.

5. To each tube add 1 c.c. of mastic reagent, mix well and allow to remain at room temperature for twelve to twenty-four hours; or in the incubator for six to twelve hours.

6. A positive reaction is indicated by the formation of a heavy precipitate which settles, leaving the supernatant fluid clear.

Mastic Reagent

Gum mastic (pure)	10 gm.
Alcohol (absolute)	100 c.c.

Dissolve the gum mastic in alcohol and filter. This can be kept as a stock solution in tightly stoppered bottles indefinitely.

One part of the above stock solution is added to nine parts of absolute alcohol; after thoroughly mixing it is sprayed into forty parts of distilled water. This final solution is opalescent and is the one used in the test. It keeps for several days.

Alkaline-Saline Solution

Sodium chlorid solution (1.25 per cent)	100 c.c.
Potassium carbonate solution (0.5 per cent)	1 c.c.

The above solutions should be made with distilled water.

EXAMINATION OF MILK

Reaction and Specific Gravity

Reaction

The reaction can be determined with litmus in the same manner as described for urine.

Specific Gravity

This can be obtained with a hydrometer in the same manner as described for urine. In cases where a small quantity is available it can be diluted and the last two figures multiplied by two or a small hydrometer can be used.

Normally the specific gravity is 1.028 to 1.032.

Fat Content of Human Milk

Cream Method

1. Fill a 10 c.c. graduate to the 10 c.c. mark with milk.
2. Allow to stand at room temperature for twenty-four hours to allow the cream to rise to the top.
3. Read off the percentage of cream formed at the top at the end of twenty-four hours and multiply this number by 0.6 to obtain the percentage of fat. Cream contains 60 per cent fat.

Normally the cream of human milk is about 7 per cent and the fat 4.2 per cent.

Babcock Test (Modified)

1. Place 5 c.c. of milk in special tube (special centrifuge tube for estimating fat in human milk).
2. Add 1 c.c. of equal parts of amyl alcohol and hydrochloric acid.
3. Add sulphuric acid (sp. gr. 1.83) slowly to the zero mark. Mix by gentle shaking and rotating.
4. Centrifuge for three minutes at 1000 revolutions per minute or until fat rises to the top.
5. The amount of fat is read on the scale at the top of the tube.
6. Each division of the scale represents 0.2 per cent.

Normally the fat in human milk is about 4.2 per cent.

Lactose Content of Human Milk

1. Acidify milk with acetic acid, boil and filter through filter paper to remove protein.
2. Determine amount of lactose present in filtrate by using Benedict's method described under urinalysis (see page 43).
3. 25 c.c. of Benedict's quantitative solution is equivalent to 0.0676 gm. lactose.

Protein Content of Human Milk

1. Dilute the milk 1:10 with distilled water; for cow's milk dilute 1:20.
2. Fill Esbach tube to mark U.
3. Add Bogg's reagent to mark R and invert several times.
4. Set aside for 24 hours.

5. Read off directly on the scale. Multiply by 2 if 1:20 dilution is employed.

Bogg's Reagent

1.	Phosphotungstic acid	25 gm.
	Distilled water	125 c.c.
2.	Concentrated hydrochloric acid	25 c.c.
	Distilled water	100 c.c.

After the phosphotungstic acid is completely dissolved, mix the two solutions and keep in dark glass bottle.

Microscopic Examination

1. Centrifuge the milk thoroughly and prepare smears of sediment.

2. Dry in the air and fix with methyl alcohol; stain with hematoxylon and eosin or Wright's stain, as for blood; or stain with Loeffler's methylene-blue.

3. Dry and examine with oil immersion lens.

4. Pus occurs as numerous large clumps of polymorphonuclear leukocytes. Erythrocytes are recognizable. Large numbers of long chains of Streptococci may be recognized.

5. Examination of cow's milk for tubercle bacilli may be made by staining smears or by guinea pig inoculation as described in the section on Bacteriology.

SECTION II

BACTERIOLOGY

SECTION II

CLINICAL BACTERIOLOGICAL METHODS

Method for Preparation of Smears of Exudates

1. Purulent material (pus) can be spread on slides in the same manner as described for making blood smears (see page 17). Avoid vigorous rubbing as the cells may be broken up so their recognition and relation to the organisms present may be difficult or impossible. This is important especially when making cyto-diagnosis or looking for intracellular organisms.

2. For eye, nose, throat and vaginal smears the material may be collected on the sterile swab and the swab in turn rolled over a slide rather than rubbed on it. Care should be taken to obtain material for smear only from the part or area affected, avoiding contamination from adjacent structures. If surface is ulcerated or membranous it is well to remove the superficial material with sterile swab and take smear from deepest surface or base of membranes.

3. Serous exudates containing few cells or organisms may be centrifuged and smears prepared from the sediment.

4. Smears the size of a nickel are large enough. Do not spread too thinly and especially if there is only a small amount of secretion.

5. Allow smears to dry in the air. Do not use the filthy and dangerous method of covering a thick wet smear of secretion on a slide with another slide.

6. Usually two smears are required; both may be placed on one slide.

7. Slides are preferred to cover glasses because they are less breakable and more easily handled.

Preparation of Cultures of Exudates

1. It is important that the infected material is collected properly so as to avoid contamination and to obtain material representative of the focal secretions.

Pus should be collected from an abscess cavity or sinus after the surrounding tissues have been cleansed with dilute tincture of iodin or alcohol.

Nasal secretions may be secured after cleansing the nasal orifice with soap and water, passing a sterile cotton swab through a nasal speculum and rubbing lightly the surfaces of the lower turbinates, septum and posterior pharyngeal wall.

Ear should be cleansed, the excess of secretion removed with sterile swab and the culture made of pus from the infected tissue.

Sputum should be collected with care. For bacteriological examination (except for tubercle bacilli) have patient brush the teeth with a sterilized brush, rinse the mouth several times with boiled water and, after swallowing several mouthfuls of water, cough and expectorate into a wide mouthed sterilized bottle. The sputum may be plated at once or washed several times in sterile Petri dishes with sterile water and then cultured.

Urine should be withdrawn with sterile catheter into sterile container after thoroughly cleansing the meatus.

Blood should be procured in same manner as described for blood cultures (see page 125), using large amounts on proper media.

2. The choice of culture media is important (see page 123). If bouillon is employed inoculate 50 c.c. of the broth with a few c.c. of the exudate. Incubate for 24 to 48 hours and make subcultures for isolation and identification of the bacteria present.

3. When organisms are numerous, as shown by preliminary examination of smears, solid media as blood agar slant or other media depending upon the organisms to be isolated may be used, in which case the material is spread over the surface of the slant. Avoid breaking up the media when inoculating as it interferes with subsequent examination; or the material may be directly inoculated on blood agar plates by taking a loopful of material and making successive streaks over the surface of the agar. After the first streak each succeeding streak will deposit less organisms,

so that the colonies developing from the last streaks should be well separated and suitable for isolating the various organisms present.

4. Another very good method is to deposit a small drop on the agar plate and spread it over the surface with a glass spreader which can readily be made by bending a small glass rod at right angles at one end using the longer end as a holder. If material contains many bacteria it may be necessary to spread several additional plates with the same glass spreader without adding any more material.

Choice of Culture Media for Pathogenic Bacteria

The choice of culture media is very important in the isolation of bacteria as organisms may grow poorly or not at all on certain media.

1. Ordinary culture media may be used for growing the following organisms:

B. coli group
B. cloacæ group
Dysentery group
Friedlander's group
Typhoid group
Gärtner group
Proteus group
Staphylococcus group
Anthrax group
B. pyocyaneus

2. Blood agar or Loeffler's blood serum are the media preferred for growing the following organisms:

Streptococcus group
Pneumococcus group
Meningococcus group
M. catarrhalis group
Hemorrhagic septicemia group
Koch-Weeks bacillus
Morax-Axenfeld diplobacillus
Bordet-Gengou bacillus
Ducrey's bacillus
Diphtheria group

3. Special media are required for the cultivation of the following:

Acid-fast group (B. tuberculosis)
Gonococcus
Spirochetes (Treponema pallidum)

Methods for Making Blood Cultures

Broth Method

1. Assemble the following:

20 c.c. Luer syringe (sterile)
Sterile needles (20 gauge)
Soap and water
Alcohol
Bichlorid of mercury (1-1000)
Tincture of iodin
Sterile gauze
Cotton
Collodion
Tourniquet
Alcohol lamp and matches
Culture media

2. Have nurse prepare the patient's arm at the elbow as follows:

Wash with soap and water.
Apply alcohol over veins at bend of elbow.
Apply bichlorid of mercury 1:1000.
The alcohol and bichlorid to be applied with sterile gauze.
Cover area with sterile gauze saturated with bichlorid until ready to take blood (at least ½ hour).

3. Apply the tourniquet; request the patient to vigorously open and close the hand.

4. Light alcohol lamp.

5. Two flasks of bouillon 300 c.c. capacity containing 200 c.c. of glucose broth should be ready for inoculation.

6. Assemble syringe. Avoid contamination.

7. Remove gauze from arm. Apply tincture of iodin over a prominent vein.

8. Make venous puncture. Avoid touching arm with fingers. If necessary to palpate vein cover finger with sterile gauze.

9. Withdraw 10 to 20 c.c. of blood.

10. Release the tourniquet; then withdraw needle from the vein.

11. Direct nurse to attend to arm and immediately remove the needle from the syringe and inoculate the two flasks of bouillon with 5 or 10 c.c. of blood.

12. Place cultures in the incubator at 37° C. for 48 hours.

13. Make smears and subcultures if cultures show any evidence of growth. Do not render a report of sterile under 5 days' incubation.

14. Employ the various methods described elsewhere in this chapter for the proper identification of the organisms found.

15. Contamination with organisms from the skin is not uncommon and should be given consideration. The presence of Staphylococcus albus is usually an indication of contamination; even the presence of pigmenting Staphylococci should not be considered as coming from the blood of the patient unless confirmed by a second blood culture.

Plating and Counting Method

1. Melt several tubes of agar (10 c.c. each) and place in boiling water in a large enamel cup with a thermometer. Keep the temperature between 40° and 45° C.

2. Have patient prepared and collect blood as just described.

3. Inoculate each tube with 1 or 2 c.c. of blood, mix thoroughly and pour into sterile Petri dishes, taking precautions to prevent contamination.

4. If the bacteria are to be counted the tubes should contain an accurately measured amount of blood.

5. After incubating plates for 48 hours examine colonies and make smears and subcultures for identification.

6. If count is desired it can be made by counting the number of colonies on several plates, using a colony counter and calculating the number of bacteria per cubic centimeter of blood.

Another Serviceable Method

1. In a sterile test tube place 1 c.c. of sterile per cent solution of sodium citrate and evaporate to dryness.

2. Collect 5 c.c. of blood as described and place in the tube, rotate the mixture with the citrate to prevent coagulation.

3. In the laboratory plate 1 c.c. or more of the blood with plain or glucose agar, boiled and cooled to 40° or 42° C.

4. After hardening incubate the plates, cover down, for 48 hours.

5. Count the colonies and estimate number of bacteria per c.c. of blood.

6. Examine colonies for identification.

Collection of Sputum for Bacteriological Examination

Sputum for bacteriological examination should be collected in a sterile wide-mouthed container and closed with a sterile stopper.

It is essential that the specimen come from the lungs and be as free as possible of contamination from the upper respiratory organs.

Sputum obtained soon after awakening in the morning is more desirable. Have patient cleanse the teeth, gums and tongue with a sterile tooth brush, rinse out his mouth with sterile saline or water and attempt to cough up some sputum (avoiding saliva).

Specimens should be placed in ice box if not examined immediately.

If expectoration is small in amount the entire amount for 24 hours should be collected.

Methods for Isolating and Cultivating Aërobic Bacteria

Shake Cultures

1. Melt 3 agar tubes by boiling.

2. Allow them to cool down to 42° C. and keep them at this temperature in a water bath until ready to inoculate.

3. Into one of the tubes place a loopful of the material to be examined; shake well to insure uniform distribution.

4. Into a second tube place a loopful from the first tube and mix thoroughly; repeat by placing a loopful into the third tube from the second, sterilizing the loop in the flame after each addition (this must be carried out rapidly as the agar may solidify before the transfers are completed and thus interfere with the next step).

5. Pour the agar from each tube into a sterile Petri dish and distribute evenly by gently rotating and tilting.

6. Allow the agar to solidify, and place the dishes in the incubator, cover side down.

7. If colonies have developed sufficiently at the end of 24 hours examine both surface and deep colonies on whichever plates they are separated sufficiently to permit of fishing and subculturing.

8. Most of the colonies with this method of plating will be deep and very difficult to differentiate by their growth.

Surface Streak Cultures

1. If the material to be examined is not liquid it should be emulsified in sterile salt solution or bouillon in a test tube, or if liquid and smears show organisms to be abundant it should be diluted.

2. Inoculate a wire loop with the material which has been emulsified or diluted and make 8 or 10 streaks over the surface of a blood agar plate or other medium which may be chosen. The loop should be allowed to rest lightly when being drawn over the agar to prevent cutting the surface.

3. After incubating a suitable length of time examine the streaks for colonies.

The first streaks may be so thick with colonies as to prevent their study, but the last ones should have colonies well separated so as to permit of their study and isolation.

Another very good method is to place a drop of the material on the surface of an agar plate and by means of a bent glass rod, which can be sterilized by flaming, spread the drop all over the agar and if many organisms are present one or more plates may be smeared without placing any more material on the rod.

Methods for Cultivating Bacteria Anaërobically

Wright's Test Tube Method

1. Boil glucose agar or gelatin to remove oxygen.

2. As soon as the media is sufficiently cool inoculate with wire loop, making a deep stab to near the bottom.

3. Flame the cotton plug and push it down into the tube so

that the top of the cotton is about three-fourths of an inch from the mouth of the tube.

4. Place a layer of pyrogallic acid about one-fourth inch thick on top of the cotton plug.

5. Add 2 or 3 c.c. of 10 per cent solution of caustic soda and insert a rubber stopper immediately.

6. Incubate.

7. To examine such cultures it may be necessary to loosen the entire culture from the sides of tube and remove it to a sterile dish where it can be cut or broken up to examine the growth. If gas is produced, cracks in the media may provide access to the growth and make it unnecessary to remove media from the tube.

Zinsser's Method for Plating Anaërobes

1. Two crystallizing dishes 3 and 4 inches in diameter and 1 inch deep are used (one larger than the other). These should be sterilized.

2. Melt glucose agar by boiling and when cooled sufficiently (45° C.) inoculate material.

3. Pour the agar into the smaller dish and allow to harden. If surface growth is desired, uninoculated agar is poured in the plate and material spread on surface after it has hardened.

4. Place dry pyrogallic acid in the bottom of the larger dish.

5. Invert the smaller dish in the larger one.

6. Quickly pour a 5 per cent solution of caustic soda into space between the sides of the two dishes (the smaller one inverted and the larger one upright) to a depth of one-half inch.

7. Immediately cover the soda solution with paraffin oil, which will act as a seal.

8. Incubate.

Vignal's Glass Tubing Method

1. Make two narrow constrictions at both ends of a section of glass tubing (one-fourth inch in diameter). Break off one end at the constriction and plug the other end with cotton. Use the cotton plugged end as mouth piece.

2. Inoculate liquid agar or gelatin.

3. Draw the medium into the tubing by suction with mouth or rubber bulb.

4. Seal the narrow constrictions at both ends in a very small flame. The medium in the tube now sealed at both ends gives very satisfactory conditions for anaërobic growth.

5. To examine such cultures after incubation it is necessary to locate colonies along the tube, file and break tube at desired points.

Stitt's Method

1. Make a deep agar stab in freshly sterilized glucose agar.

2. Place the culture and required amount of pyrogallic acid (1 gm. per 100 c.c. of space) in a salt mouth bottle.

3. A rubber stopper perforated with a glass tube to which rubber tubing is attached is firmly pushed in.

4. Connect rubber tubing with vacuum pump and in 5 or 10 minutes all air will be exhausted. Then clamp the rubber tubing with Hoffman clamp and screw it up tightly. Disconnect from pump.

5. Insert the tubing into a graduate filled with 10 per cent caustic soda. Unscrew the clamp and allow necessary amount of soda solution to be drawn in (1 gm. per 10 c.c. of space). Screw the clamp up tight.

6. Incubate.

Methods for Staining Bacteria

Loeffler's Methylene-Blue

1. Make thin smear of material to be examined on slide.

2. Dry in air and fix with gentle heat.

3. Cover smear with stain and allow to stand for 2 to 5 minutes.

4. Wash with tap water, blot and examine with oil immersion lens.

5. Methylene-blue does not stain very intensely and there is very little danger of overstaining. It is a very good stain to use when studying the morphology of organisms and is used especially in the examination of cultures for diphtheria bacilli.

Formula of Stain

Saturated alcoholic solution of methylene-blue.....	30 c.c.
Potassium hydroxid 0.01 per cent solution.....	100 c.c.

Carbolfuchsin (Ziehl-Neelsen's solution)

1. Make thin smear on slide or cover glass.
2. Dry in air and fix by gentle heat.
3. Cover the smear with the stain, using it either diluted or undiluted depending upon the intensity of staining desired. To dilute, cover the slide with water, then drop on the slide a drop or two of the stain or dilute 1 to 10 with water beforehand.
4. Allow the stain to act for a minute or two. Heat may be applied if intense staining is desired.
5. Wash with tap water, dry and examine with oil immersion lens.
6. This is a very strong stain and overstaining may occur. The intensity of staining is best regulated by using proper dilution of stain, the degree of heat and time stain is allowed to act.

Formula of Stain

Method 1

Distilled water	10 c.c.
Carbolic acid (crystals)	5 gm.
Alcohol	10 c.c.
Fuchsin	1 gm.

Method 2

Carbolic acid, 5 per cent solution	100 c.c.
Fuchsin, sat. alcoholic solution	10 c.c.

Gram's Method

1. Make thin smear of material on glass slide or cover glass.
2. Dry in air and fix with gentle heat.
3. Cover smear with gentian-violet solution (Weigert's) for two minutes.
4. Wash with water.
5. Cover with Gram's iodin solution for one minute.
6. Pour off excess and wash with acetone until smear ceases to lose color (few seconds).
7. Wash with water.
8. Counterstain with 1-10 solution of carbolfuchsin for one minute.
9. Wash in water.
10. Dry and examine with oil immersion lens.

11. The Gram-positive organisms will appear dark violet or purple, the Gram-negative organisms will be colored a faint pink by the fuchsin. Occasionally capsules may be demonstrated with this method.

Weigert's Gentian-Violet

Solution No. 1

Gentian-violet	2 gm.
Anilin oil	9 c.c.
Alcohol (95 per cent)	33 c.c.

Solution No. 2

Gentian-violet	2 gm.
Distilled water	100 c.c.

Mix 1 c.c. of solution No. 1 with 9 c.c. of No. 2 and filter. The stock solutions Nos. 1 and 2 keep indefinitely, but when mixed to make staining solution it does not keep well and becomes unsatisfactory in about two weeks. It is well to mix and filter only enough of Nos. 1 and 2 to last two weeks.

Gram's Iodin Solution

Iodin	1 gm.
Potassium iodid	2 gm.
Distilled water	300 c.c.

Method for Staining Acid-Fast Bacilli

1. Prepare thin smears of material on slide or cover glass.
2. Dry in air and fix by gentle heat.
3. Cover smear with undiluted carbolfuchsin, gently heating to steaming for five minutes (do not boil).
4. Wash in tap water.
5. Decolorize with acid alcohol (2 per cent HCl in 95 per cent alcohol); 15 per cent sulphuric acid or 30 per cent nitric acid in water.
6. Wash in water.
7. Counterstain with Loeffler's methylene-blue for 2 minutes.
8. Wash in water.
9. Dry and examine with oil immersion lens.
10. The acid-fast organisms will appear red; all other organisms and cells will appear blue. This method of staining is extensively used for detecting the tubercle bacillus in sputum and

other material. Spores will also retain the red stain, and this method may be used to demonstrate their presence.

Neisser's Stain for Diptheria Bacilli

1. Prepare smear in usual manner, fix with heat and place in solution No. 1 for 2 or 3 seconds.
2. Wash in tap water.
3. Place in solution No. 2 for 3 to 5 seconds.
4. Wash, dry and mount.
5. The bacilli may stain uniformly brown or may show at one or both ends a dark blue, round body. True diphtheria bacilli usually show the blue bodies, while the pseudotypes show few if any.

Neisser's Solution No. 1

Methylene-blue	0.1 gm.
Alcohol	2.0 c.c.
Glacial acetic acid	5.0 c.c.
Distilled water	95.0 c.c.

Dissolve the methylene-blue in alcohol and add to the acetic acid and water.

Neisser's Solution No. 2

Bismarck brown	0.2 gm.
Water (boiling)	100.0 c.c.

Dissolve the stain in boiling water and filter through filter paper.

Giemsa's Method

1. Fix smears in neutral methyl alcohol for one minute.
2. Cover slide with Giemsa stain diluted as follows: Add one drop of a 1 per cent solution of potassium carbonate to 10 c.c. of distilled water and then add 10 drops of the stain (one drop of stain to each c.c. of alkaline distilled water).
3. Allow the stain to act for one-half to three hours or overnight (the longer the better).
4. Pour off stain and wash in running tap water for 2 or 3 minutes.
5. Dry with filter paper.
6. Nuclear substance—blue, red or azure.

Bacteria—dark purple.
Metachromatic granules—azure.
Cytoplasm of protozoön—blue.

The stain can be purchased ready for use from any laboratory supply house.

Methods for Staining Spirocheta Pallida (Treponema Pallidum) and Other Spirochetes

Fontana's Method

1. Wash lesion with alcohol; allow to dry and gently scrape with scalpel or rub with gauze until lymph from the corium may be obtained. Avoid obtaining blood. Place drop of lymph on clean slide and spread very thin. Dry without fixing.
2. Cover smear with Ruge's solution for 1 minute.
3. Wash with water.
4. Cover slide with alcohol and ignite.
5. Cover smear with mordant and warm gently until steam arises and then allow to act for 30 seconds.
6. Wash with water.
7. Cover smear with Fontana's solution and gently warm until steam arises and allow to act for 30 seconds.
8. Wash dry and mount.
9. The spirochetes are stained dark brown or black.

Ruge's Solution

Pure acetic acid	1 c.c.
Formalin (40 per cent)	2 c.c.
Distilled water	10 c.c.

Mordant

Tannic acid	5 gm.
Distilled water	10 c.c.

Fontana's Solution

To a 5 per cent silver nitrate solution add ammonia drop by drop with a capillary pipet until a dark precipitate forms and redissolves. Then add more silver nitrate solution until the solution is slightly cloudy.

Tilden's Method

1. Suspend tissue scrapings in a small amount of fixative solution and allow to stand at least 5 minutes (the longer the better).

If the material is small in amount place a drop of fixative on clean slide and add drop of material and let stand for 5 minutes.

2. Make very thin smears of this mixture on clean slides and dry in the air.

3. Cover the smear with a saturated solution of gentian-violet or fuchsin.

4. Almost immediately wash in running tap water and dry in the air.

Tilden's Fixing Solution

Phosphate buffer solution (pH 7.6)	9 parts
M/15 Na_2HPO_4	88 parts
M/15 KH_2PO_4	12 parts
Formaldehyde solution	1 part

India Ink Method

1. Take one loopful of secretion from a chancre or other material to be examined and place on one end of glass slide.

2. A small quantity of drawing ink is mixed with the secretion on the slide. This can well be done by dipping a match stick or toothpick in the ink and transferring a small amount to the slide and mixing it with the secretion.

3. After mixing make a smear in same manner as described for blood (see page 17).

4. Allow to dry and examine with oil immersion lens.

5. The spirochetes will appear as white spirals against a dark background.

Methods for Staining Spores

Acid-Fast Stain

The method described for staining acid-fast bacteria (see page 139) may be employed for staining spores. By this method spores are stained red and bacillary bodies blue.

Abbott's Method

1. Cover film with Loeffler's alkaline methylene-blue.
2. Heat to boiling from time to time for 1 to 2 minutes.
3. Wash with water.
4. Decolorize with 2 per cent nitric or hydrochloric acid alcohol until blue disappears.

5. Wash with water.
6. Counterstain with 10 per cent aqueous solution of eosin.
7. Wash and dry.
8. Spores are stained blue; bacillary bodies pink and red.

Methods for Staining Capsules

Gram's Stain

For Gram's method, see page 137.

India Ink Method

For this method, see page 145.

Welch's Method

1. Cover film with glacial acetic acid for few seconds.
2. Drain and replace with anilin gentian-violet. Drain and again replace with anilin gentian-violet. Repeat until all acid has been replaced by gentian-violet solution.
3. Wash in a 1 or 2 per cent solution of sodium chlorid and mount in same.
4. The capsule stains pale violet.

Hiss's Copper Sulphate Method

1. Grow organisms in ascitic fluid or serum medium or mix with drop of serum and prepare smears.
2. Air dry and fix with heat.
3. Cover preparation with 5 or 10 per cent water solution of gentian-violet or fuchsin and heat for few seconds until steam arises.
4. Wash dye off with 20 per cent solution of copper sulphate.

Huntoon's Method

1. Mix the organisms with a small drop of nutrose solution on a slide and spread the film; dry in air (do not fix).
2. Cover with stain for thirty seconds.
3. Wash in water and dry.

Nutrose Solution

Nutrose (sodium caseinate)	3 gm.
Water	100 c.c.

Cook the solution for 1 hour in Arnold sterilizer and add 0.5 per cent carbolic acid. Place in tubes without filtering.

Huntoon's Stain

Carbolic acid (2 per cent aqueous solution)	100.0 c.c.
Acetic acid (1 per cent aqueous solution)	1.0 c.c.
Lactic acid (concentrated)	0.5 c.c.
Carbolfuchsin	1.0 c.c.
Basic fuchsin (sat. alcoholic solution)	1.0 c.c.

Methods for Staining Flagella

Preparation of Film

1. Place enough of an 18 hour culture in a few cubic centimeters of filtered tap water to produce slight cloudiness. This may be used immediately or placed in an incubator for one or two hours to permit slight development.

2. Place a very small drop of the suspension on a clean slide; spread and allow to dry quickly. The less the suspension is manipulated the better. If drying is slow the bacteria may shed their flagella; if handled roughly they will be broken off.

Pittfield Method. (Muir's Modification)

1. Cover film with mordant and steam gently for 1 minute.
2. Wash well in water for about 2 minutes.
3. Dry in the incubator (do not blot with paper or cloth).
4. Apply stain and heat gently for 1 minute.
5. Wash with water, dry and mount.

Mordant

Tannic acid (10 per cent aqueous solution)	10 c.c.
Saturated aqueous solution mercuric chlorid	5 c.c.
Saturated aqueous solution alum	5 c.c.
Carbolfuchsin	5 c.c.

Allow the precipitate to settle or it may be centrifuged. This solution will only keep about one week.

Stain

Saturated aqueous solution of alum	10 c.c.
Saturated alc. solution gentian-violet or carbolfuchsin	2 c.c.

This stain does not keep over two days.

Methods for Staining Organisms in Tissues

Bacteria may be demonstrated in sections of tissues prepared by the paraffin or other methods and stained with methylene-blue,

Gram method, acid-fast method for tubercle and lepra bacilli, etc. Spirochetes may be stained by the Levaditi, Noguchi or Warthin method. The technic employed by us is described in *Arthur's Practical Laboratory Methods and Histologic Technic* (F. A. Davis Company, Philadelphia, Pa.).

Methods for Studying Unstained Bacteria

By Hanging Drop

1. Smear a little vaselin around the edge of the hollow space on a hanging drop slide.

2. Place a loopful of fluid containing the bacteria in the center of a cover glass and immediately invert it over the hollow space on the slide, resting it on the vaselin.

3. Press the cover slip gently to spread the vaselin and to make a perfect seal. The purpose of the vaselin is to keep the cover slip from sliding and the fluid from evaporating.

4. Examine with either 1/6 objective or oil immersion lens. If examining only for motility the lower power usually suffices. Care should be taken when focusing not to break the cover glass. The light should be considerably reduced with the diaphragm.

By Dark Field Illumination

1. Apparatus necessary: strong illuminating lamp such as a small arc lamp or high power incandescent lamp, funnel stop to be placed in 1/12 objective to cut out rays which interfere, and a dark field apparatus (special substage condenser).

2. Remove substage condenser from microscope and adjust dark field condenser in its place.

3. Insert funnel stop in the 1/12 objective.

4. Center the apparatus with low power lens by getting concentric rings on the upper surface of the dark field condenser and adjusting by means of the three centering screws on edge of the condenser until the rings are parallel with the circle of the microscopic field.

5. Place a small drop of material to be examined on a scrupulously clean slide (1.45 to 1.55 mm. thick) and covered with clean cover glass (avoid air bubbles).

6. Place large drop of oil on top of condenser and put slide

on stage so the oil forms a contact with the under surface of the slide.

7. Place oil on top of cover slip and examine with 1/12 objective. Examination can also be made with low and high power objectives.

Methods for Studying Biological Properties of Bacteria

Pigment Production

1. Many bacteria are capable of producing pigments of various colors which are of interest on account of their value in identifying these organisms. The pigmenting organisms of particular value on account of their pathogenic properties are:

Staphylococcus citreus—yellow pigment.
Staphylococcus aureus—orange pigment.
B. pyocyaneus—blue and green pigment.
B. prodigiosus—red pigment.

2. Under certain conditions chromogenic bacteria may fail to produce pigment, *e.g.,* under anaërobic conditions, B. pyocyaneus does not produce its pigment; many conditions interfere with pigment production; occasionally a part of a culture will show pigment while other parts fail to show any. At present the cause of these variations is not well understood.

3. To demonstrate pigment production inoculate the surface of various solid media and incubate under following conditions: at 37° C. and at 20° C., aërobically, exposed and unexposed to light.

Liquefaction of Gelatin and Coagulated Blood Serum

1. The liquefaction of gelatin and coagulated blood serum (Loeffler's) is due to the production of a proteolytic enzyme by certain bacteria.

2. The demonstration of the production of this enzyme is of value in the identification of bacteria. It is to be kept in mind, however, that there may be considerable difference in production of this ferment by different cultures of the same variety of bacteria.

3. To demonstrate this proteolytic activity of bacteria inoculate

a tube of liquid gelatin; shake well to distribute organisms, and after incubating for 24 to 48 hours place in ice chest or in glass of ice water. If digestion has taken place the medium will remain liquid while a sterile control will solidify. Some organisms liquefy slowly; therefore incubation should continue for at least 7 days when liquefaction cannot be demonstrated earlier.

Coagulation of Milk

Certain bacteria when grown in milk cause its coagulation. In most cases this is due to formation of acid as the result of fermentation of the lactose present. A rennin-like enzyme has been found to be produced by some bacteria which is capable of coagulating the milk independent of the acids.

To demonstrate this property of bacteria, inoculate a culture of plain milk. Incubate and examine daily. Coagulation usually occurs within 1 to 7 days with whey formation.

Formation of Acid and Gas from Carbohydrates

1. Acid and gas may both be produced by many bacteria in culture media containing sugars or higher alcohols. Some, however, produce only acid, in which case the acid formed is usually pure lactic or acetic acid. About one-third of the acid-producing bacteria produce gas.

2. To demonstrate the formation of acid inoculate the medium containing a carbohydrate or higher alcohol and an indicator. The medium should be neutral or slightly alkaline in reaction. Incubate and if acid is formed the color of the indicator will change accordingly; if litmus is used the change will be from blue to red.

3. Another method is to determine the reaction of the medium before and after incubation by titration.

4. To demonstrate gas the medium should be such that the gas formed will not escape but collect as visible bubbles in the medium or the medium may be placed in fermentation tubes so the gas formed will collect in the closed arm.

5. Media commonly used for demonstrating acid or gas formations are: litmus milk, Hiss's serum water media containing carbohydrates, Russell's double sugar, glucose or other sugar broths in fermentation tubes, etc.

Indol Production

Certain bacteria, particularly those belonging to the colon and proteus groups, produce indol by decomposition of protein. The recognition of this substance in cultures may be used as a means of differentiating certain closely related organisms. The technic is as follows:

1. Inoculate peptone solution or plain broth and incubate two to seven days.
2. Add 6 or 8 drops of concentrated sulphuric acid to the culture.
3. If both indol and a nitroso body are present a violet pink coloration will be produced. This is the *cholera red* reaction.
4. Should no change take place add 1 c.c. of a 1 : 10,000 dilution of sodium nitrate.
5. If indol is present a pink color will be produced.
6. By using trypsinized broth (Rivas) the culture may be examined in less than 48 hours.

Voges-Proskauer Reaction

1. Inoculate fermentation tubes containing 2 per cent glucose Dunham's peptone solution with organism to be tested.
2. Incubate 3 days.
3. Add to the culture 2 or 3 c.c. of strong caustic potash solution.
4. A positive reaction is indicated by the development of a pink color on exposure to the air. This test is of some value in differentiating closely related organisms.

Reduction of Nitrates

Some bacteria are capable of reducing certain substances. A typical example is the changing of nitrates to nitrites. The following test may be used to demonstrate this property of bacteria:

1. Grow organism in broth containing nitrate for four or five days.
2. To 4 c.c. of the culture add 2 c.c. of the reagent.
3. Place the mixture in warm water for 15 or 20 minutes.
4. A positive reaction is indicated by the development of a pink color.

Reagent

Solution No. 1

Naphthylamin	0.1 gm.
Distilled water	20.0 c.c.
Acetic acid, 25 per cent	105.0 c.c.

Dissolve the naphthylamin in the distilled water by heat. Allow to cool, then filter and add the acetic acid.

Solution No. 2

Sulphanilic acid	0.5 gm.
Acetic acid dil. 1 to 16	105.0 c.c.

Mix equal parts of solution Nos. 1 and 2 to obtain reagent for use in test. These solutions should be kept separate and sufficient mixed from time to time as needed.

Key of Regional Distribution of Pathogenic Bacteria After Park, Williams and Krumwiede

Cerebrospinal fluid

Micrococcus meningitidis.
Streptococcus (including pneumococcus group).
B. influenzæ group.
B. tuberculosis group.

Pericardial and pleural fluids

Streptococcus (including pneumococcus group).
B. mucosus (capsulatus) group.
B. influenzæ (hemoglobinophilic bacilli) group.
B. tuberculosis group.

Peritoneal fluid

B. coli group.
Streptococcus group.
B. tuberculosis group.
Yeasts, molds, amebæ.

Lungs

Streptococcus (including pneumococcus group).
Staphylococcus group.
B. mucosus (capsulatus).

B. influenzæ group.
B. tuberculosis group.
B. pertussis group.
Nocardia, yeasts, molds, amebæ.

Nose and throat

Streptococcus and staphylococcus groups.
B. diphtheriæ group.
B. influenzæ group.
B. mucosus group (micrococcus group).
B. fusiformis.
B. tuberculosis.
Yeasts, molds, amebæ.
Tr. pallidum and other spirochetes.

Sputum

Same organisms as those found in the lungs, nose and throat.

Eye

Streptococcus (including pneumococcus group and staphylococci).
Group of hemoglobinophilic bacilli.
Gonococcus group.
B. diphtheriæ group.
B. Morax-Axenfeld group.
B. tuberculosis group.
Yeasts and molds.

Feces

B. coli (including B. alkaligenes and B. lactici).
B. typhosus group.
B. diphtheriæ group.
Gram-positive anaërobes.
Yeasts, molds, amebæ.
B. tuberculosis.
Vibrio cholera group.
B. pestis.
B. anthracis.
Many forms whose importance has not been worked out.

Urine

B. coli group.
Streptococcus (kidney).
M. gonorrhœæ.
B. typhosus.
B. tuberculosis.
Molds, flagellates.

Pelvic organs

M. gonorrhœæ.
Streptococcus.
B. tuberculosis.
Treponema pallidum.
Certain anaërobes.
Many other forms probably much less important.

Blood

Streptococcus (including pneumococcus) group.
B. typhosus group.
Trypanosome group.
Group of malarial organisms.
B. anthracis.
Sp. recurrentis.

Brain

Micrococcus group.
Strepto-pneumococcus group.
Group of hemoglobinophilic bacilli.
B. tuberculosis group.
Tr. pallidum.
Malarial organisms.
Rabies organisms.
Yeasts, molds, etc.

Key for the Identification of Pathogenic Cocci

Gram-Positive Cocci

1. Streptococci—cocci form chains.
   S. hemolyticus—clear zone of hemolysis surrounds colony on blood agar.

S. non-hemolyticus—blood agar media not altered.

S. viridans—greenish zone around blood agar colonies.

2. Pneumococci—cocci oval or lance shaped, usually arranged in pairs; occasionally in short chains. Greenish colony on blood agar.

3. Staphylococci—cocci form grapelike bunches.

Staph. pyogenes albus—no pigment produced.

Staph. pyogenes aureus—orange or golden pigment.

Staph. pyogenes citreus—yellow pigment.

Gram-Negative Cocci

1. Gonococcus—requires special media for growth.
2. Meningococcus—grows on blood agar.
3. M. catarrhalis—grows on plain agar at room temperature.

The following table shows the fermentation reactions of various Gram-negative cocci:

TABLE III

Gram-Negative Cocci	*Glucose*	*Maltose*	*Saccharose*	*Levulose*
Meningococcus	+	+	—	—
Parameningococcus..	+	+	—	—
Gonococcus	+	—	—	—
M. flavus I	+	+	+	+
M. flavus II	+	+	—	+
M. flavus III	+	+	—	—
M. catarrhalis	—	—	—	—
M. pharyngis siccus.	+	+	+	+

Bacteriological Diagnosis of Staphylococcus Infections

1. Inoculate cultures with pus or collections of purulent material. In cases of abscess or other infections in which the organisms are very likely to be present in pure culture, agar slants may be used. If other organisms are likely to be present, as in mucous membrane or skin affections, an agar plate may be inoculated.

However, if this is not available the slant may be used and usually proves satisfactory, as Staphylococci are seldom overgrown by other organisms.

2. Media to use—any of the ordinary media.

3. The cultures should be incubated 24 hours.

4. Smears made from suspicious colonies should be stained by Gram's method.

5. Note if pigment is produced. If not expose cultures to daylight at room temperature for another 24 hours.

6. The two varieties usually encountered are the Staphylococcus albus and the Staphylococcus aureus.

The aureus is found about twice as often in pathological processes. The albus variety is the common cause of stitch abscess, it is less virulent than the aureus and seldom produces pyemia. Both varieties may be present in the same infection.

Staphylococcus albus and aureus are normally present in the skin and mucous membranes.

Blood cultures in Staphylococcus sepsis are more often negative than in Streptococcus sepsis. This is probably due to the fact that Staphylococcus, after entering the blood stream, localizes rather rapidly.

Some of the conditions that may be caused by Staphylococci are abscesses, carbuncles, acne pustules, bacteremia, osteomyelitis, endocarditis, pyelitis, pyelonephritis, etc.

The mere presence of the Staphylococcus in cultures from a lesion does not by any means indicate that it is the cause of the infection. As noted above, they are normally present in the skin and mucous membranes and may contaminate cultures.

Bacteriological Diagnosis of Streptococcus Infections

Direct Smears of Exudate

1. Make thin smear of exudate to be examined on a cover glass or slide.

2. Fix and stain by Gram's method (see page 137).

3. Streptococci appear as blue or blue-black cocci in chains. By this method type III pneumococcus may be differentiated by its rather large capsule. Note whether chains are long or short, particularly when examining nose or throat smears, as the varieties normally present in this region are usually of the short chain

variety, whereas the longer chain variety (10 to 20 cocci) are more likely to be virulent types. The abundance of organisms and whether phagocytosis has taken place are also to be noted.

Cultural Methods for Exudates

1. Blood agar is the medium of choice; glucose broth excellent.
2. Exudate collected in manner described on page 121.
3. Slants may be inoculated when plates are not available. The slant or broth culture to be sent immediately to the laboratory for transference to plates (see page 131).
4. After incubating plates 24 to 48 hours, examine.
5. Streptococcus colonies are small and discrete. The types may be differentiated by the colonies on blood agar. The water of condensation should be examined when slants are used.

   Streptococcus hemolyticus—clear zone of hemolysis around colony.

   Streptococcus non-hemolyticus—no hemolysis.

   Streptococcus viridans—greenish colonies.

6. The pneumococcus produces small greenish colonies not unlike the Streptococcus viridans and at times it may be difficult to differentiate these two organisms without special tests (see page 175).

Blood Cultures in Sepsis

Make blood cultures as described on page 125. Several flasks should be inoculated with 10 or 15 c.c. of blood.

Occurrence in Health.—Streptococci occur normally in the nose, throat and skin. The finding of Streptococci in cultures from these parts does not necessarily mean that they are producing disease. The short chain varieties are the ones normally found, but it is not uncommon to find long chain Streptococci in healthy throats and furthermore short chain varieties may at times prove very virulent.

Occurrence in Disease.—Streptococci do not typically produce pus; the inflammatory changes produced are usually diffuse, and necrosis common. The Streptococcus is often present as a secondary infection in such diseases as tuberculosis, smallpox, diphtheria and typhoid fever. The Streptococcus viridans is associated with tooth abscesses, tonsil and focal sinus infections, ulcerative endocarditis, chorea, gastric and duodenal ulcers, cholecystitis and appendicitis. Streptococcus hemolyticus is claimed as the cause

of scarlet fever and is usually the cause of non-diphtheritic anginas, Ludwig's angina, puerperal fever and erysipelas. Streptococci have been found the cause of primary importance in the following diseases: acute abscesses, impetigo, cellulitis, acute peritonitis, pneumonia, bronchopneumonia, periostitis, synovitis, otitis media, mastoiditis, enteritis, some cases of rheumatic fever, meningitis, pleurisy and empyema, septic thrombosis following mastoiditis, etc.

Bacteriological Diagnosis of Pneumococcus (Diplococcus Pneumoniæ) Infections

Smear Method

1. Wash a portion of the sputum several times with normal salt solution.
2. Choose for examination a portion of the more tenacious sputum and spread in thin smear on slide. Spinal or other fluids (pleural, etc.) should be centrifuged and smears made from sediment unless they are purulent.
3. Dry in air and fix with gentle heat.
4. Stain by Gram's method (see page 137).

The Gram-positive pneumococci will be recognized as dark violet or purple lancet shaped diplococci; the Gram-negative organisms will be colored a faint pink or red. Sometimes with the Gram stain a capsule will be seen surrounding the pneumococci.

Culture Method

1. With platinum loop streak out one or two blood agar plates with material to be examined. If sputum is to be examined a small portion should be first washed in sterile salt solution.
2. Place plates in incubator for 24 or 48 hours.
3. Examine characteristic colonies for Gram-positive diplococci.

The pneumococcus colony on blood agar is small and greenish with no hemolysis. It may be confused with the Streptococcus viridans as the colonies are very similar and it often forms small chains. Bouillon cultures of pneumococci are bile soluble, Streptococci are not. The pneumococci ferment inulin; Streptococci do not.

Animal Inoculation

1. Wash a bean-sized mass of sputum and emulsify in a small amount of sterile salt solution.

2. Inoculate into peritoneal cavity of a mouse.

3. As soon as mouse shows evidence of illness, which is usually in 5 to 8 hours, it is killed.

4. Make smears and cultures from heart's blood and peritoneal exudate.

5. Usually the pneumococcus can be obtained in pure culture either from the heart's blood or peritoneal exudate.

The mouse is very susceptible to pneumococcus infection and this organism when injected into the peritoneum multiplies more rapidly than other organisms contained in sputum or other exudates and is not destroyed by the animal's natural defenses as other organisms may be.

Types of Pneumococci.—There are four types of pneumococci, or rather groups designated as types I, II, III and IV. They differ in virulence and immunological reactions. Strains belonging to group IV are the least virulent and are the ones commonly found in normal throats and are called the "mouth strains." The most virulent are types II and III; the latter has a tendency to grow in chains and develops a very large capsule and at one time was known as the Streptococcus mucosus.

A method for determining types by the agglutination test is given in the section on serological methods (see page 255).

Pneumococcus in Health.—Normally they may be found on the mucous membranes of the nose and throat of most persons. The types commonly found are the less virulent ones belonging to group IV. However, virulent types are encountered especially in persons who have been in contact with cases of pneumonia.

Pneumococcus in Disease.—The pneumococcus is the causative agent in the vast majority of cases of lobar pneumonia as well as most cases of bronchopneumonia or bronchitis. Pleurisy developing secondary to pneumonia is also the result of invasion with this organism. Other conditions which may be caused by these organisms are meningitis, empyema, otitis media, endocarditis, pericarditis, rhinitis, tonsillitis, conjunctivitis, keratitis, arthritis, osteomyelitis, orchitis, salpingitis, peritonitis, appendicitis, etc.

Differentiation of Streptococci from Pneumococci

Bile Solubility Test

1. To a 24 hour bouillon culture of organisms to be tested add 0.5 to 1.0 c.c. of ox bile which has been filtered and sterilized (to 10 c.c. of bouillon add 1 c.c. of bile).
2. Place the cultures in incubator for one-half hour.
3. Streptococcus cultures will remain unchanged. Pneumococcus cultures clear up (organism is bile soluble).

Fermentation of Inulin

1. Inoculate Hiss serum water medium to which inulin has been added.
2. Pneumococci will ferment inulin with the formation of acid. Streptococci do not ferment inulin.

Bacteriological Diagnosis of Gonococcus (Neisseria Gonorrheæ) Infections

Smear Method

1. Make two thin smears of exudate.
2. Dry in air and fix with heat.
3. Stain one slide with Loeffler's methylene-blue (see page 135) and the other with Gram's method (see page 137).
4. Wash, dry and mount.

The gonococci will appear as biscuit or coffee bean shaped diplococci. They are Gram-negative and therefore stain red or pink with this method.

In the peracute stage before the exudate becomes profuse, the organisms may be extracellular but become intracellular during the acute stage when the exudate is at its height. At this stage it is common to find many organisms gathered within one leukocyte while other cells in the immediate neighborhood have none. Later, when the condition becomes more chronic the organisms become less numerous and are extracellular.

Vaginal Washings

1. Fill vagina with 1 : 4000 bichlorid of mercury in normal salt solution by means of a sterile bulb or syringe.
2. Recover fluid and transfer to a centrifuge tube.
3. Centrifuge at once and pour off supernatant fluid.

4. Prepare smears of sediment for gonococci as just described.

This method of examination is used in the diagnosis of infantile vaginitis and often yields better results than direct smears.

Examination of Urine for Gonococci

1. Have patient present himself with full bladder.
2. Have ready two sterile bottles or centrifuge tubes.
3. Allow patient to void part in one bottle or tube and part in the second. While urinating in second tube press the patient's prostate with the finger. The first bottle or tube will contain material from the anterior urethra and the second will likely contain secretions from the posterior urethra and prostate.
4. Centrifuge the urine collected and examine the sediment for gonococci. In case the above method is negative the secretions should be stimulated by massage or injections of silver nitrate. Gonococcus vaccine has been recommended as a means of provoking an increase of the number of gonococci in chronic cases.

Cultural Method (Park and Williams)

1. Add a small amount of rich sterile ascitic fluid to tube containing swab (if patient is at a distance from the laboratory the ascitic fluid is sent in a separate tube).
2. Transfer the exudate to ascitic fluid with sterile swab. Mix well by stirring the fluid well with swab.
3. Withdraw swab from ascitic fluid and smear over surface of ascitic agar (1-4) plates containing glucose. (Smear in strokes radiating from center of plate.)
4. With platinum loop streak other plates of the same media with ascitic fluid suspension.
5. Incubate at 36° C. for 24 hours.
6. Look for gonococcus-like colonies; if present make smears and stain by Gram's method and examine for gonococcus-like organisms. Then make subcultures on ascitic, hydrocele or blood streaked agar.

The colonies of gonococci on ascitic agar appear as minute, irregular, dewdrop spots. Frequent transplants are necessary to keep the culture alive. For differentiation from other Gram-negative diplococci, see page 165.

Bacteriological Diagnosis of Meningococcus (Neisseria Intracellularis) Infection

Smears of Spinal Fluid

Owing to the fact that meningococci often undergo rapid autolysis, the specimen should be examined as soon as possible after fluid has been taken from patient.

1. Centrifuge the fluid at high speed. If the fluid is very cloudy direct smears may be made without centrifugation.
2. Make thin smears from the sediment.
3. Stain with Gram's stain; stain a second slide with methylene-blue.
4. The finding of Gram-negative cocci, either intracellular or extracellular, indicates meningococcus infection. However, this should be confirmed by culture.

Cultures of Spinal Fluid for Meningococcus

1. As soon as possible after the fluid is withdrawn from patient place 1 c.c. on each of several blood agar or Loeffler's blood serum plates or tubes and spread over surface.
2. Incubate at 37° C. for 24 hours.
3. If no growth occurs allow to incubate another 24 hours.
4. Examine for meningococcus-like colonies. If present make smears and stain by Gram's method and make subculture for further identification (see page 165).

Examination for Carriers

1. The material for examination should be collected from the nasopharynx, particularly the posterior part.
2. By means of a bent applicator or West tube material is collected from nasopharynx. Avoid touching soft palate or other structure.
3. Immediately inoculate the material on blood or sheep serum dextrose agar and place in incubator at once. The plates should be warm when inoculated. This can be done by keeping them in the incubator ready for use.
4. Meningococcus-like cultures should be differentiated from other Gram-negative cocci, particularly M. catarrhalis, M. flavus and M. pharyngis siccus (see sugar fermentation tests on page 165).

General Bacteriological Examination of Spinal Fluid

1. Collect fluid in sterile containers or tubes.
2. Examine as soon as possible after collection.
3. Centrifuge at high speed (one hour in the case of clear fluids).
4. Prepare smears and cultures from sediment.
5. Stain smears with Gram's stain. Clear fluids should also be stained for acid-fast bacilli (see page 139) and inoculated into guinea pigs if tuberculosis is suspected.
6. Meningococcus, B. influenza } Gram-negative.
   Pneumococcus, Streptococcus, Staphylococcus } Gram-positive.
   Tubercle Bacilli Acid-fast.

The table on page 183, taken from Park, Williams and Krumwiede, gives the differential points in spinal fluids.

Bacteriological Diagnosis of M. Catarrhalis (Neisseria Catarrhalis) Infections

Smears

1. Prepare smears from mucous membrane.
2. Fix and stain by Gram's method.

The Micrococci catarrhalis appear in pairs and sometimes fours. They are Gram-negative. They are coffee bean in shape, resembling gonococci but slightly larger.

Cultures

1. Spread or streak exudate from mucous membrane on blood agar plates. Slants may be used if plates are not available or the patient is some distance from laboratory. The organism will grow on plain agar but not so well as on blood agar.
2. Incubate plates for 24 hours.
3. The colonies are small gray or yellowish white, circular with irregular borders. Make smears and subcultures from suspicious colonies.

For further differentiation from other Gram-negative cocci, especially the meningococcus, see page 165.

TABLE IV

	Pressure	Amount in c.c.	Appearance	Cytology	Bacteria	Albumin	Globulin	Animal Inoculation
Normal	Normal	5–10	Clear	Very few cells	Sterile	±	—	Negative
Meningismus	Increased	10–100	Clear	Very few cells	Sterile	±	—	Negative
Infantile paralysis	Increased	20–100	Clear; sometimes slight fibrin web	Early polynucleosis; later lymphocytosis up to 98 per cent endothelial cells	Sterile	+−++	+++	Negative or pneumonia in 4 weeks
Tuberculous meningitis	Increased	30–120	Clear; fibrin web	Lymphocytosis up to 98 per cent	Tubercle bacilli	++−+++	++−+++	Tuberculosis in 4 weeks
Epidemic cerebrospinal meningitis	Increased	5–120	Cloudy	Polynucleosis up to 98 per cent	Meningococcus	++−++++	++−++++	
Meningitis due to other organisms	Increased	20–100	Cloudy	Polynucleosis up to 98 per cent	Infecting organisms	++−++++	++−++++	

These organisms may be found in the secretion of normal mucous membrane and in certain diseased conditions may be abundant. At times they may excite catarrhal inflammation and pneumonia.

Identification of Spore-Bearing Aërobic Gram-Positive Bacilli

Anthrax group—B. anthracis.
Mycoides group—B. mycoides, B. ramosus.
Subtilis group—Hay bacillus.

Table V

	Anthrax Group	Mycoides Group	Subtilis Group
Gelatin stab	Branching along stab	Branching along stab	No branching
Bouillon	No membrane non-motile	Thick membrane slight motility	Membrane actively motile
Virulence	Virulent	Non-virulent	Non-virulent

B. anthracis is the only one of this group which is pathogenic for man and can always be differentiated by demonstrating its pathogenicity by subcutaneous inoculation of guinea pigs.

Bacteriological Diagnosis of Anthrax (B. Anthracis)

Examination of Pustule by Smear

1. Inoculate plain agar tubes, or better, plates, with material from the pustule. When taking material from a pustule before excision, avoid rough manipulation which might cause the bacteria to be expressed into the blood circulation.
2. Make smears and stain by Gram's method.

The anthrax bacillus is Gram-positive, short chains, ends are square and may be capsulated. A diagnosis may be made from the smear alone but this should be confirmed by culture.

Examination of Pustule by Culture

1. Inoculate plain agar.
2. Incubate twenty-four hours.

The growth is rapid. The colonies are grayish white, flat and spreading, the edges showing filamentous projections. Under the

low power they appear like waves of hair or, better, a Medusa head. This organism is commonly confused with the hay bacillus. It is non-motile; B. subtilis is highly motile.

By Blood Cultures

The organisms may appear in the blood and especially in severe cases just before death. Blood cultures have definite value in prognosis and when positive indicate active therapeutic measures. Human beings may die from a toxemia or as a result of bacteremia.

If blood cultures are made, at least 15 to 20 c.c. should be collected and cultured in plain broth, as described on page 125.

Identification of the Spore-Bearing Anaërobic Bacilli (Stitt)

1. Rods very little swollen by centrally situated spores:
   (*a*) Motile: B. sporogenes.
   (*b*) Non-motile: B. welchii (capsule).
2. Spores tend to be situated between center and end:
   (*a*) Tends to form chains: Vibrion septique.
   (*b*) Single or in pairs: B. botulinus.
3. Spores situated at end of rod: drum-stick sporulation: B. tetani.

A study of the pathogenic effects of the organisms of this group is of considerable assistance in their identification. The following table from Lehrman and Neumann gives an outline of the effects produced by subcutaneous inoculation:

1. No particular symptoms at site of inoculation; absorption of the soluble toxin causing:
   (*a*) General symptoms of tetanus: B. tetani.
   (*b*) Botulism-poisoning symptoms: pupillary symptoms; paralysis of tongue and pharynx. Cardiac and respiratory failure: B. botulinus.
2. Local symptoms marked at site of inoculation. Hemorrhagic emphysematous edema.
   (*a*) Motile
      (*a*) digests blood serum: B. sporogenes.
      (*b*) no action on blood serum: Vibrion septique.
   (*b*) Non-motile
      B. welchii.

Bacteriological Diagnosis of Tetanus (Clostridium Tetani)

Smears

1. Make smears from each wound or points of suppuration.
2. Stain with carbolfuchsin.

Look for the typical spore-bearing or drum-stick forms. These are rarely found by direct smear. Note the presence of other bacteria.

Culture

1. Pieces of tissue, pus, etc., are dropped into glucose bouillon to which is added a small piece of fresh tissue. See method for culturing anaërobic organisms, page 131.

2. If material is probably contaminated with tetanus spores and other organisms, prepare an emulsion in broth, heat to 80 ° C. for thirty minutes in a water bath (kills all except spore-bearing bacilli) and culture. For identification see page 187.

Inoculation

1. Inoculate a mouse or guinea pig subcutaneously with pus, bits of tissue, etc., from wound.

2. If tetanus bacilli or spores are present in the material inoculated the animal will develop, in from 1 to 4 days, symptoms of tetanus. In mice and guinea pigs the muscles nearest the point of injection are first affected. At autopsy, no changes are found except a hemorrhagic spot at site of injection or suppuration due to other bacilli present in the inoculum.

Bacteriological Diagnosis of Malignant Edema (Clostridium Œdematis-maligni)

By Smear

Prepare smears of the frothy sour serous fluid and stain by Gram's method. *B. maligni edema* (*Vibrion septique*) is Gram-amphophil; may show very narrow and faint capsule; occurs singly or in short chains. Wet preparations show motile bacilii.

By Culture

Boil a tube of glucose agar and cool to 40° C. Inoculate with exudate and cover with sterile vaselin, paraffin oil or molten agar (the bacillus is an obligate anaërobe). Incubate 24 to 48 hours.

Gas and acid are produced. Long bacilli in smears found end to end in pairs and chains. Spores may be found. No capsules; motility present. For identification see page 187.

By Animal Inoculation

Cultures or exudates are highly pathogenic for young guinea pigs, mice and rabbits.

Bacteriological Diagnosis of Gas Wound Infections (Clostridium Welchii)

1. With sterile applicator obtain material from wound. Use a separate applicator for each part of wound examined. (Always examine several parts, especially the deeper parts.)
2. Place applicator in sterile test tube for purpose of carrying or storing until media are inoculated.
3. Boil glucose agar tubes for 10 minutes and cool quickly to 42 ° C.
4. Inoculate agar by rubbing swab in it. See that material on swab is well distributed through the agar.
5. Solidify agar in ice box.
6. Incubate at 37 ° C.
7. If gas bacilli (B. welchii) are present, gas will form rapidly after 12-19 hours. The tube will be filled with gas bubbles and broken up.
8. Make smears from culture and stain by Gram's method. Look for rather large Gram-positive bacilli. For differentiating the Gram-positive anaërobes, see page 187.

Bacteriological Diagnosis of Diphtheria (Corynebacterium Diphtheriæ)

By Smears

1. By means of a cotton swab or platinum loop secure a small bit of exudate or membrane to be examined (usually from nose and throat).
2. Make a thin smear on a slide or cover glass.
3. Stain with Loeffler's methylene-blue for one or two minutes. Neisser's stain is also recommended for demonstrating granules (see page 141).

4. Wash with water, dry and examine with oil immersion objective.

The diphtheria bacilli will appear as rather short, irregular staining, occasionally club-shaped bacilli arranged parallel or around a common center like the spokes of a wheel. They can be divided into three main types—solid, barred and granular.

By Cultures

1. With cotton swab or platinum loop secure specimen as described above.

2. Smear over surface of Loeffler's blood serum medium.

3. Incubate at 37 ° C. for 18 to 24 hours. In 18 to 24 hours the diphtheria bacilli produce a luxuriant, yellowish white or creamy growth, usually in the form of isolated colonies.

4. From this make a smear on a glass slide or cover glass and stain as described above.

The smear will often show a pure culture, owing to the fact that in the first 18 to 24 hours the diphtheria bacilli outgrow the other organisms. For differentiation from diphtheroids, see page 195.

Bacteriological Diagnosis of B. Xerosis (Corynebacterium Xerosis) Infection

1. This is the special name given to the diphtheroid bacillus of the eye.

2. The bacillus may be found on the normal conjunctivæ; it sometimes produces a low grade chronic conjunctivitis.

3. Smears of conjunctival secretion stained by Loeffler's methylene-blue or Gram's method show solid bacilli of typical grouping and morphology.

4. Cultures on Loeffler's blood serum or blood agar show luxuriant growths of large, solid, Gram-positive bacilli of typical grouping.

5. Cultures are non-virulent for guinea pigs.

6. Cultures usually do not ferment the sugars in Hiss-serum water medium, although some cultures ferment some sugars and especially saccharose. For differentiation from B. diphtheria, see page 195.

Determination of the Virulence of Diphtheria Bacilli

1. Wash off 18- to 24-hour pure culture with 6 c.c. sterile salt solution and inject under skin of the abdomen of an 8- to 10-ounce guinea pig; or, if culture is in bouillon, inject subcutaneously 2 c.c. of culture (do not use pigs weighing more than 10 ounces).

2. A control pig may be injected with the same dose of culture plus 1 c.c. of antitoxin (500 units).

3. If the organisms are virulent diphtheria bacilli, the first pig will die in 4 days or show edema at the site of injection. Smears and cultures from the site of inoculation will show B. diphtheriæ. A characteristic edema at the site of injection and congestion of the adrenal glands will be seen. The control pig should show no ill effects.

Differentiation of Diphtheria Bacilli from Hoffman's Bacillus (Corynebacterium Hoffmanni) and B. Xerosis

B. Hoffmanni and B. xerosis differ from true diphtheria bacilli in that they are shorter, thicker, often not curved, rarely give the blue dot staining at both ends, produce little or no gas in sugar media and are non-virulent.

TABLE VI

Fermentation Sugars

Species	Dextrose	Dextrin	Saccharose
B. diphtheriæ...	+	+	—
B. xerosis.....	+	—	+
B. Hoffmanni...	—	—	—

Bacteriological Diagnosis of Vincent's Angina (Spironema Vincenti and Fusiformis Dentium)

1. Make smear on glass slide.
2. Dry in air.
3. Fix by passing through flame four times.
4. Cover with carbolfuchsin diluted 1:10 with water; heat gently and stain for two minutes.

5. Wash in water and dry.

6. Examine with oil immersion lens for fusiform bacilli and spirilla.

Bacteriological Diagnosis of Tuberculosis (Mycobacterium Tuberculosis)

Smear Examination of Sputum

1. Spread out suspected sputum between two glass plates, as described for general examination of sputum (see page 95), or pour into a Petri dish and pick up with sterilized platinum wire small white or yellow caseous particles; if none are present, choose for examination some of the thicker yellowish or greenish portions.

2. Make smears on glass slides.

3. Stain with Ziehl-Neelsen's carbolfuchsin for acid-fast organisms, as described on page 139.

The tubercle bacilli will appear as red solid or vacuolated straight or slightly curved rods; other bacteria and cells are stained blue.

Smear Examination of Urine

1. Shake the specimen of urine thoroughly, fill two centrifuge tubes, 50 c.c. capacity, and centrifuge (if smaller tubes are used more will have to be used or the centrifuging repeated until sediment from at least 100 c.c. of the urine has been collected).

2. Mix the sediment from both tubes or the sediment from 100 c.c. of urine. (If sediment contains crystals they should be dissolved by very small amounts of either ammonium hydroxid if acid, or acetic acid if alkaline, diluted with water and centrifuged).

3. By means of platinum loop transfer sediment to slides.

4. Spread out in thin films and dry.

5. Fix by gentle heat.

6. Stain for acid-fast bacilli by Ziehl-Neelsen method described on page 139.

It may be necessary to add a drop of Mayer's egg-albumin to the sediment on the slide, before spreading, to facilitate adherence.

Smear Examination of Feces

1. Make thin smear of feces on slide or cover glass; if blood or mucus is present in the feces, this will be more apt to contain bacilli.

2. Dry in air, fix and stain for acid-fast bacilli, in the same manner as outlined for sputum examination.

Smear Examination of Pleural and Spinal Fluids

1. Collect and prepare smears as described above for urine; if a coagulum has formed, remove and tease out on a slide with pins.

2. Stain and examine for acid-fast bacilli as described on page 139. Prolonged search is generally required.

3. For differential diagnosis of spinal fluid, see page 183.

Note: Tubercle bacilli are the only acid-fast bacilli encountered in spinal fluids.

Cultural Method of Isolating Tubercle Bacilli

1. Tubercle bacilli in exudates or other material contaminated with other organisms are best isolated by injecting the material into a guinea pig and then obtaining material aseptically from the tuberculous lesions. Such material will contain many more tubercle bacilli and none of the contaminating organisms. Otherwise the tubercle bacilli grow slowly and may be readily overgrown by contaminating organisms.

2. Inoculate media containing blood serum or egg yolk as Petroff's medium or Dorsett's egg medium with original material or from a tuberculous gland or spleen of the previously inoculated guinea pig. The material should be thoroughly disintegrated and rubbed over the media. Paraffin the stoppers to prevent drying of the medium.

3. Examine the cultures after several weeks' incubation (2 to 4) for growth of tubercle bacilli. At first the growth appears as small grayish-white points or scales. Later it becomes membranous, looking irregular, and becomes more or less elevated. Examine suspicious growths.

Detection of Tubercle Bacilli by Animal Inoculation

1. Sputum should be washed in sterile salt solution. If urine is to be examined, collect sediment from 100 c.c. Feces should be treated with antiformin (see page 201).

2. Weigh two guinea pigs and note their weights. Also note their color or otherwise mark them for further identification.

3. Inoculate each pig subcutaneously with the sediment from 50 c.c. of urine, a bean-sized portion of sputum or the washed sediment of feces after treatment with antiformin.

4. Bloch recommends damaging the inguinal lymph glands by squeezing between the finger, and injection of the material into these damaged glands.

5. Examine the animals each week for symptoms of tuberculosis.

Weigh them and examine site of inoculation for tubercles or tuberculous ulceration, also superficial lymph glands or enlargements.

6. If the animals show physical signs of tuberculosis at the end of two weeks, one may be killed and the presence of tuberculosis confirmed, in which case the other animal may be destroyed and examined. If necropsy of first animal fails to reveal tuberculosis, allow the other to live for four to six weeks, then destroy, examine and report positive or negative findings.

7. Should neither pig show any signs of tuberculosis, both should be allowed to live four weeks before destroying.

It sometimes happens that specimens of urine contain organisms other than tubercle bacilli in large numbers which cause the death of the inoculated animals in a few days requiring a repetition of the test.

8. Enlarged glands or tissue from other organs (spleen) may be examined for tubercle bacilli by making smears and staining. If acid-fast bacilli are not found, the tissues should be examined histologically for tuberculosis before a negative report is warranted.

9. Never base a negative report alone on the absence of enlarged external glands; the internal glands and spleen require examination.

Detection of Tubercle Bacilli by Antiformin Method

This is especially useful where sputum contains only a few organisms. Examination of the entire 24 hours' sputum is suggested.

1. Place equal parts of sputum and 50 per cent antiformin solution in small beaker.

2. Warm gently for ten minutes, stirring occasionally to insure complete liquefaction.

3. Dilute with 3 volumes of sterile water to reduce specific gravity of the solution.

4. Centrifuge for 10 to 30 minutes; pour off supernatant fluid; fill tube up with solution; centrifuge; continue until all of the fluid has been centrifuged.

5. To sediment left, after pouring off supernatant fluid, add sterile distilled water, centrifuge and pour off fluid.

6. By means of a platinum loop transfer sediment to cover glass.

7. Make smears and stain for acid-fast bacilli as described for sputum (see page 139).

If the sediment does not adhere to the slide, apply a thin smear of Mayer's albumin or raw egg-albumin to the slide and spread the sediment.

Bacteriological Diagnosis of Leprosy (Mycobacterium Lepræ)

By Examination of Skin Lesions by Smears

1. Scrape a nodule or spot deeply with scalpel until epidermis is removed and serum exudes.

2. Prepare smears of the serum and stain by Ziehl-Neelsen's method for acid-fast bacilli (see page 139). Twenty per cent sulphuric acid is preferred by some instead of acid alcohol for decolorizing. The lepra bacilli are less resistant to acid alcohol than the tubercle bacillus. Be careful not to carry decolorization too far.

By Smears of Nasal Mucus

1. If coryza does not exist give patient 60 grains of potassium iodid to produce a drug coryza.

2. Prepare smears of scrapings from ulcerations on nasal septum if present, or from the nasal mucus.

3. Stain and examine by method described above.

The above examination is sometimes of value in the diagnosis of leprosy and particularly in nerve leprosy.

Methods for Differentiating the Gram-Negative Aërobic Bacilli Which Grow Poorly or Not at All on Ordinary Culture Media (Hemophilic Bacteria)

All the organisms belonging to this group require either blood, serum, or both in the media. They produce minute dewdrop colonies. The following key is taken from *Stitt's Practical Bacteriology, Blood Work and Animal Parasitology*:

Do Not Grow On Ordinary Media.—Require blood agar (hemophilic bacteria), serum agar, or blood serum. Minute dewdrop colonies.

1. Influenza bacillus (Pfeiffer). Requires blood media.
2. Koch-Weeks bacillus (conjunctivitis). Serum agar best medium. Many, however, regard hemoglobin as necessary for growth.
3. Müller's bacillus of trachoma. Like Koch-Weeks bacillus, but easier to cultivate.
4. Morax-Axenfeld diplobacillus of conjunctivitis. Grows well and produces little pits of liquefaction on Loeffler's blood serum.
5. Bordet-Gengou bacillus of whooping-cough. Does not grow on Loeffler's serum. Requires blood or ascitic fluid agar. Original isolation should be on glycerin-potato agar.
6. Ducrey's bacillus (soft chancre). Requires media rich in blood or serum. Forms chains.
7. Bacillus of tularemia. Grows only on coagulated egg yolk, cystin agar or blood agar containing sterile animal tissue.

Bacteriological Diagnosis of Acute Contagious Conjunctivitis (Pink-eye)

By Smears

1. Prepare smears from conjunctival secretion and stain by Gram's method (see p. 137).
2. Examine for Koch-Weeks bacillus (*Hemophilus conjunctivitidis*) which is a small Gram-negative bacillus slightly larger than B. influenzæ. Usually intracellular and extracellular. In acute cases these organisms are usually present in large numbers; they end to occur in shoals near the pus cells.

By Cultures

1. Inoculate blood agar slants if plates are not available, or better a mixture of glycerin agar and ascitic fluid with the exudate collected from bottom of conjunctival sac.

2. After 48 hours' incubation examine the cultures for very small dewdrop-like colonies of Gram-negative bacilli.

3. For differentiating from other Gram-negative bacilli, see page 205.

Bacteriological Diagnosis of Morax-Axenfeld (Hemophilus Lacunatus) Conjunctivitis (Angular Conjunctivitis)

1. Prepare smears and cultures on blood agar or Loeffler's serum.

2. Stain slides by Gram's method and look for Gram-negative bacilli which are a little longer but much thicker than the Koch-Weeks bacillus. They usually occur in pairs or short chains.

3. Examine the cultures after 24 hours for Gram-negative bacilli, which, when grown on Loeffler's blood serum, produce little pits. See page 205 for difference from the Gram-negative bacilli.

Bacteriological Diagnosis of Whooping-Cough (Bordet-Gengou Bacillus, Hemophilus Pertussis)

1. Examine sputum during onset of disease, as the Bordet-Gengou bacillus, considered as the cause of whooping-cough, will only be found during the early stages.

2. Prepare smears and stain by Gram's method (see page 137). Look for small oval Gram-negative bipolar bacilli resembling somewhat B. influenzæ (see page 205).

Bacteriological Diagnosis of Chancroidal Infection (B. Ducrey, Hemophilus Ducreyii)

Smears made from the lesion, particularly before ulceration sets in, may be of diagnostic value. These should be stained with carbolfuchsin and Gram's stain. The organisms stain best with the fuchsin, show polar staining, are small Gram-negative and grow in chains (see page 205).

Identification of Gram-Negative Bacilli Which Grow Well on Ordinary Culture Media

The Organisms Included Here Are as Follows:

B. coli group—B. coli communis and B. coli communior.
B. cloacæ group—B. cloacæ.
B. lactis aërogenes group—B. aërogenes.
Dysentery group—B. dysenteriæ of Shiga, Flexner, etc.
Friedländer's group—B. pneumoniæ, B. capsulatus, B. rhinoscleromatis.
Typhoid group—B. typhosus.
Gärtner group—B. enteritidis; B. paratyphosus.
Proteus group—Proteus vulgaris.
Chromogenic group—B. pyocyaneus, B. prodigiosus.

Note: The chromogenic group are readily identified by their property of producing pigments (see page 153).

On Endo or Conradi Medium

Plates containing either of these media are used for the isolation of, and differentiation of, members of the colon, typhoid and intermediate groups of Gram-negative bacilli. The surface is inoculated either by streaking or spreading (see page 131).

The colon, cloacæ and lactic aërogenes groups produce red colonies.

The typhoid and other groups produce bluish or colorless colonies.

The suspected colonies are fished and subcultures subjected to further study.

On Russell's Double Sugar Medium

This medium contains 1 per cent lactose, 0.1 per cent glucose and an indicator. It is useful for differentiating members of the colon-typhoid-paratyphoid groups in pure culture. The medium is inoculated by passing the loop over the surface of the slant and then making a stab culture in the butt of the media. The principle is that bacilli growing on the surface (aërobically) will only ferment sugar in the amounts over 0.1 per cent, but when growing

in the butt (anaërobically) they ferment the small amount of glucose to obtain oxygen. The reaction is indicated by the formation of gas bubbles or change in the color of the media.

Colon group—slant acid; butt acid and gas.

Paratyphoid group—slant unchanged; butt acid and gas.

Typhoid group—slant unchanged; butt acid only.

B. alcaligenes—slant unchanged; butt unchanged.

Four Culture Method

Considerable information may be obtained for the identification of pure cultures by inoculating the following media: litmus milk, bouillon (for demonstrating motility), glucose medium (for demonstrating acid and gas) and gelatin. It should be remembered that many members of the various groups can only be definitely identified by more extensive study of cultural characteristics than given here or by serological tests.

TABLE VII

Organisms	B. Coli Group	B. Cloacæ Group	B. Lactis Aërogenes Group	Dysentery Group	Friedländer's Group	Typhoid Group	Paratyphoid Group	Proteus Group
Litmus milk	Pink	Pink	Pink	No change	No change	No change	No change	No change
Motility	Motile	Motile	Non-motile	Non-motile	Non-motile	Motile	Motile	Motile
Glucose	A. & G.	A. & G.	A. & G.	Sl. A.	A. & G.	A.	A. & G.	A. & G
Gelatin	No liq.	Liq.	No liq.	No liq.	No liq.	No liq.	No liq.	Liq.

A = acid; G = gas; Liq. = liquefaction.

Bacteriolgical Examination of Urine for B. Coli (Escherichia Coli), B. Typhosus (Eberthella Typhi) and B. Paratyphosus (Salmonella Paratyphi and Schotmuelleri)

1. Collect urine in sterile container by catheterization under aseptic conditions.
2. Inoculate glucose broth with 2 to 5 c.c. of the urine.
3. Incubate for 24 to 48 hours.
4. Make smear and stain by Gram's method.
5. Examine for Gram-negative bacilli; if present, plate on endo media (see page 209) or inoculate slant and butt of tube of Russell's double sugar medium.
6. Make subcultures and identify by cultural or serological

methods. See four tube method on page 211 and agglutination tests on page 247.

7. If no growth occurs in 48 to 72 hours, specimen was sterile.

Bacteriological Examination of Feces for Typhoid, Paratyphoid and Dysentery Bacilli (Eberthella Dysenteriæ)

1. Specimen should be collected in clean container, or better, a sterile one.

2. If specimen is solid emulsify with a small amount of sterile water in a test tube.

3. With a platinum loop streak two or three well hardened plates of endo medium (see page 209).

4. Incubate for 24 hours at 37° C.

5. Examine plates for suspicious colonies; colon group produces red colonies, typhoid and paratyphoid groups bluish white or colorless colonies.

6. Fish 5 or 10 suspicious colonies and make suitable subcultures for identification.

7. Identification can be accomplished in either of two ways: serologically by agglutination tests of bouillon cultures with immune serum (see page 247) or bacteriologically by cultural and biological reactions (see four tube culture method on page 211).

Preparation of Blood Cultures for Typhoid and Paratyphoid Fevers

Blood cultures are of particular value in the early diagnosis of typhoid during the first week or ten days.

1. Make blood cultures as described on page 125.

2. Inoculate glucose bouillon flasks containing about 200 c.c. of medium, with 5 to 10 c.c. of blood from patient.

3. Incubate for 24 to 48 hours.

4. Make smears and subcultures for identifying Gram-negative bacilli (see page 209).

Bacteriological Diagnosis of B. Friedländer (Encapsulatus Pneumoniæ) Infection

By Smears

1. Prepare smears of infected material as described on page 119.

2. Stain with Gram's stain and capsule stain.

3. Examine for Gram-negative coccoid bacilli which have a tendency to grow in chains and are usually surrounded with a distinct wide capsule.

Smears alone cannot be depended upon and findings should be confirmed by cultures.

By Culture

1. Inoculate agar slants or plates (the organism grows well on ordinary media).

2. Examine colonies which are glistening and mucoid in appearance and are tenacious when removed by platinum loop; stain for Gram-negative bacilli which often in cultures show capsule formation.

3. Make subcultures and identify by methods given elsewhere, particularly the four tube method on page 211.

4. Normally the Friedländer's bacillus is occasionally found in the upper respiratory tract and accessory sinuses.

In disease it may be the cause of both bronchopneumonia and lobar pneumonia, otitis media, meningitis, pleuritis, pericarditis, and bacteremia. It may localize and cause abscesses and is occasionally the cause of inflammation of the eye.

Examination of Feces for Spirillum Choleræ (Vibrio Comma)

1. Inoculate peptone water with feces and incubate 6 to 12 hours.

2. Examine smears from surface growth for actively motile vibrios. Stain with 10 per cent carbolfuchsin (see page 137). Conduct indol test for cholera red reaction.

3. Prepare agar plates for typical heaped-glass colonies.

4. If vibrios are numerous, make a microscopic agglutination test with immune serum (see section on Blood, page 247).

5. If vibrios are not found, another tube of peptone water may be inoculated from the first and, after incubation, examined; or inoculate a selective medium as alkaline egg-agar and, after incubation, select typical colony and apply agglutination test.

6. The vibrios are very actively motile and Gram-negative.

Bacteriological Diagnosis of B. Pyocyaneus (Pseudomonas Aeruginosa) Infection

1. Inoculate plain agar slants or plates with material to be examined.

2. Incubate at 22° C. for 24 to 72 hours. The organism grows well at 37½°, but pigment production is more marked at the lower temperature.

3. Examine for greenish colonies or greenish growth on agar tubes. This coloration is due to the formation of pigments which not only colors the growth, but also diffuses through and colors the medium. Two pigments are produced: one soluble in water which is green and the other soluble in chloroform and is blue (pyocyanin).

4. To differentiate B. pyocyaneus from other fluorescing bacteria examine an agar culture for pyocyanin by agitating it with chloroform; a blue coloration indicates its presence. The other fluorescing bacteria do not produce pyocyanin.

5. B. pyocyaneus is widely distributed in air and water, also frequently found in feces. It is commonly found in association with other pathogenic organisms and has been found to be the sole cause of intestinal disorders, middle ear inflammation and cystitis.

Bacteriological Examination of Bile and Duodenal Fluids

1. Two c.c. of fluid are drained directly into flasks containing broth. The medium should be favorable for the growth of Streptococci and special precautions taken to prevent contamination from the upper digestive tract.

It is well to stress the difficulty of isolating Streptococci in the presence of other rapidly growing organisms, particularly the colon bacillus; when Streptococci are seen in fresh smears a special effort should be made to get it out, as the Streptococci are of immense importance clinically.

2. Incubate the flasks for 24 hours. Should no growth occur, continue the incubation for another day or two.

3. Make smears of the broth and examine after staining by Gram's method.

4. If the culture appears to contain only one organism, make

subcultures on blood-agar slants. If more than one organism appears to be present, make blood-agar plates.

5. Isolate and identify organisms present.

Laboratory Diagnosis of Ringworm

1. Examine hairs in same manner as described under favus (see below).

2. There are three different species of fungi causing ringworm:

Trichophyton megalosporon endothrix:—the cause of ringworm of the beard (Barber's Itch). It is composed of spores of various sizes lying in chainlike formation up and down the hair shaft and mycelium which are not found within the hair.

Trichophyton microsporon:—the cause of ringworm of the scalp and differs from the preceding as follows: spores smaller and round, irregularly grouped around follicular portion of hair, mycelia often found within the hair.

Microsporon furfur:—the cause of the condition known as tinea versicolor and is composed of spores arranged in clusters which are highly refractive and resemble droplets of oil. The mycelia are not usually branched. Found readily in the scales scraped from the affected skin. Stains better than the favus or other ringworm fungi.

Laboratory Diagnosis of Favus

1. Macerate a favus crust with a few drops of water or, better, a 10 per cent solution of sodium or potassium hydrate.

2. Place material on slide, cover with cover glass and gently press out. Hairs from affected areas should be examined in the same manner.

3. Examine with 2/3 objective.

4. The fungus (Achorion schönleinii) appears as threads (mycelia) which may be simple or segmented and as irregularly shaped bodies (conidia) which are in chains or encapsulated.

Staining Method for Favus and Ringworm Fungi

1. Material collected by scraping the skin should be mixed with egg-albumin or serum and smeared on slide and dried.

2. Treat with alcohol and then with ether (to reduce fatty material).
3. Cover with Gram's iodin for 1 to 2 minutes.
4. Dry.
5. Stain with anilin gentian-violet for 1 to 5 minutes.
6. Dry in air.
7. Cover with Gram's iodin for 1 or 2 minutes.
8. Clear with anilin oil.
9. Wash in xylol.
10. Mount in balsam or lactophenol (phenol 20 c.c.; lactic acid 20 c.c.; glycerin 40 c.c.; water 20 c.c.).

Cultural Method for Favus and Ringworm Fungi

1. Inoculate Sabouraud's maltose or dextrose agar slants or plates. Do not seal tubes or place rubber caps over mouth. The reaction of the media should be about + 2.
2. Incubate at 30° to 36° C.
3. Growth may not become visible until after one week or even longer. The culture is seldom pure and subsequent transplantations have to be made to obtain pure cultures.

Laboratory Diagnosis of Blastomycosis

Smears

1. Prepare smears on slides or cover glasses.
2. Stain with methylene-blue or Jenner's stain (see page 19).
3. Examine for yeast cells occurring in torular form (budding).

Cultures

1. Blastomycetes grow well on ordinary media with reaction neutral, slightly acid or alkaline.
2. Inoculate agar slants or plates.
3. Incubate for a week or ten days.
4. Examine for rather large cells resembling yeast cells and multiplying by budding.

Methods for the Diagnosis of Sporotrichosis

Direct Method

The method outlined for favus may be used.

The material for examination should be collected from the nodules or the pus if discharging.

The direct method is not very satisfactory, as the organisms can rarely be demonstrated by this method. Cultural method should always be employed.

The fungus appears as narrow filaments with grapelike clusters of oval spores at the end.

Cultures

1. Inoculate material same as for other fungi (see page 221).
2. Growth appears in 2 to 7 days, as small white colonies which later become larger circular and characteristically raised and ringed.
3. Examine unstained preparations of suspicious growth.
4. The fungus appears as narrow filaments with grapelike clusters of oval spores at the end.

Laboratory Diagnosis of Actinomycosis (Actinomyces Hominis)

Direct Examination of Pus

1. Spread material on this film over the bottom of a Petri dish and look for small white or yellowish granules about the size of a pin head. These granules are colonies of actinomyces.
2. By means of a platinum loop place a granule on a slide and cover with cover glass; press out gently.
3. Examine with 2/3 objective or oil immersion lens. The actinomycotic granule (colony) will be made up of filaments radiating from the center. The terminal ends of the filaments around the periphery will appear bulbous or club shaped giving the appearance of rays of light radiating from a center (ray fungus).

Stained Smears

1. Prepare thin smears of material, particularly the granules just described.
2. Stain with Gram's stain. The central mycelium of the colony will stain Gram-positive; the clubs or bulbous ends will not stain with Gram's.

Cultures

1. Inoculate deep glucose agar stabs or bouillon.
2. Incubate 37° C.

3. The growth is dry and chalky. In bouillon, growth only takes place at the bottom of the tube. This organism grows better under anaërobic conditions; it is therefore advisable to inoculate the media according to the method described on page 131.

Laboratory Diagnosis of Thrush

1. Remove a small portion of white patch from mucous membrane.
2. Press out well between a cover glass and slide.
3. Examine with 2/3 objective.
4. The thrush fungus endomyces (monilia) albicans appears as a network of filaments which may be segmented and branching. Epithelial cells, leukocytes and débris will also be present.

Laboratory Diagnosis of Aspergillosis

Direct Method

1. Mount the material scraped from the lesions by the method described for ringworm and favus (page 219).
2. Examine for mycelia showing fructification at the end in the case of the violet or gray-violet varieties, and filaments and scattered spores in the case of the white, black and red varieties.

Cultures

Employ same method as described for culturing favus and ringworm (see page 221).

Laboratory Diagnosis of Amebiasis

Amebic Dysentery (Entameba Coli and Histolyticus)

1. Examine stool as soon as possible after being passed. If immediate examination is not possible, collect stool in warmed container and keep warm until examined. Stool should be free of urine.
2. Choose for examination flakes of mucus or bits of tissue.
3. Place drop of material on warm clean slide and cover with cover glass.
4. Examine on the warm stage with 1/6 or 2/3 objective, shutting down the light considerably.
5. The amebæ are distinguished from other cells, such as leukocytes, by their marked motility, larger size, greenish color,

hyaline ectoplasm, faint nucleus, vacuoles and the presence of red cells or tissue cells in cytoplasm.

Amebæ of Pyorrhœa Alveolaris (Entameba Gingivalis)

1. Wipe away superficial material, as pus from around root of tooth.
2. With tooth pick obtain material by scraping the bottom of the pus pocket.
3. Emulsify in salt solution or saliva or clean warm slide and cover with cover glass.
4. Examine as described above.

Laboratory Diagnosis of Rabies

By Smears of Brain Tissue

1. Prepare smears from the cornu ammonis (hippocampus major). The best results will be obtained by making impression smears by first removing a section of the hippocampus, making a smooth cut across one end. Pick up with forceps, holding it near cut end. Bring cut surface in contact with clean slide, press lightly against slide and then remove. Sufficient material adheres to slide for examination. Make several of these impressions on the same slide (6 or 8).
2. Place slide in methyl alcohol for 2 or 3 minutes.
3. Remove from alcohol and allow to air dry.
4. Cover smear with the following stain and steam gently:

Sat. alcoholic sol. of basic fuchsin.....	3 drops
Loeffler's methylene-blue	2 c.c.
Distilled water	10 c.c.

This stain will keep fairly well in ice chest.

5. Wash with water and dry.
6. Locate ganglion cells with 2/3 objective and then examine the cells with oil immersion for the Negri bodies, which appear as round or oval bodies, stained magenta and containing blue granules. Nerve cells blue; red cells are salmon or yellow.

By Tissue Sections

This method is used when direct smears fail to show Negri bodies.

By Animal Inoculation

1. Macerate small portion of brain tissue in salt solution.
2. Inoculate a rabbit subdurally with a few drops of the brain emulsion.
3. In positive cases the animal will develop symptoms of rabies in the dumb form (paralysis) in about three weeks. It is, however, necessary to keep the animals under observation for 60 to 90 days before reporting negative results.
4. If the animal develops symptoms and dies, the brain should be removed and examined for Negri bodies.

Laboratory Diagnosis of Kala-Azar

By Direct Smear

1. Make blood smear from peripheral circulation as described under blood (page 17).
2. Stain with Wright's or Giemsa's stain.
3. Examine with oil immersion lens for the Leishman-Donovan bodies in the large mononuclear types of cells. The organisms will be found enclosed in leukocytes, particularly the large mononuclears, endothelial cells and macrophages. Rarely are they found in the polymorphonuclear neutrophils or free in the blood. They are most abundant in the spleen, liver, lymph glands and bone marrow. They are also found in some of the other organs of the body and in ulcers of the mucous membranes.

They appear as oval or pear-shaped, resembling a cockle shell, and have a granular protoplasm with oval nucleus which stains well.

The examination of the peripheral blood cannot be depended upon, as the organisms are rarely found by this method. Therefore if this method gives negative results, then material obtained by liver or splenic puncture or from superficial lymph glands should be examined.

By Cultural Method

1. Collect blood by vein puncture in citrate saline solution (sodium citrate 1.5 per cent in saline solution), placing a small amount of the solution in the syringe before filling with blood. For details, see method for blood culturing, page 125.

2. Inoculate special media as the N.N.N. medium, or simple citrated blood.

3. Incubate at 22° to 25° C.

4. Examine from time to time for slender, narrow, oval organisms having a nucleus, a micronucleus and a simple anterior flagellum.

5. The Leishmania infantum grows well in the water of condensation of the N.N.N. medium. The Leishmania may be grown better in simple citrated blood.

By Formol-gel Test

1. Place a drop of clear serum from the patient on a slide.

2. Place a few drops of liquor formaldehyd in a small watch glass.

3. Invert the slide with serum over the watch glass.

4. A positive reaction is indicated by solidification of the serum. The serum becomes opaque, still like jelly and adheres to the slide. This reaction is positive in cases of kala-azar, but has been reported to occur in other diseases. Therefore it cannot be considered as specific.

Bacteriological Examination of Milk

Cultural Method for Counting Bacteria

1. Into three sterile flasks or bottles place the following amounts of sterile distilled water: 99 c.c. in the first; 90 c.c. in the second, and 99 c.c. in the third. Mark the containers, 1, 2 and 3 with wax pencil.

2. Shake the specimen of milk well and with a sterile pipet place 1 c.c. in the first flask which contains 99 c.c. of water, thus making a dilution of 1:100. Shake well.

3. Remove 10 c.c. from flask 1 and place in flask 2 containing 90 c.c. of water, thus making a dilution of 1:1000.

4. Remove 1 c.c. from flask 1 and place in flask 3 containing 99 c.c.

5. Melt 6 tubes of litmus-lactose agar by boiling. Allow them to cool down to 42° C.

6. Into six sterile Petri dishes place 1 c.c. of each dilution in each of two plates.

7. Add liquid culture medium, mix thoroughly and allow to harden.

8. Place the plates in the incubator for 48 hours at 37° C.

9. Count the number of colonies on the plates, using a colony counter.

10. Multiply the number of colonies by the dilution to obtain the number per c.c. or original milk.

11. Do not count plates showing over 300 or under 30 colonies as the results in such cases are not dependable.

Preparation of Bacterial Vaccines

1. Proper collection of infected material is very important (see method for preparing cultures of exudates on page 121).

2. Inoculate proper media for isolation of organism or organisms present (see page 123). A preliminary smear of the material often indicates the nature of the infection and aids in the choosing of proper media. If more than one organism is present the material should be plated.

3. Inoculate solid media with a pure culture of the organism isolated. For freely growing organisms, as the Staphylococci, use two agar slants; for slowly growing organisms, as Streptococci and pneumococci, use five or six tubes or, better in some cases, inoculate serum or hormone bouillon tubes instead of solid media.

4. Incubate culture for 24 hours.

5. Make smears from cultures to detect contamination.

6. Cover the growth on one of the tubes with sterile salt solution, taking precautions against contamination. Bring the organism into suspension either by shaking or with a platinum loop.

The suspension from the first tube should be transferred to the second tube and the growth removed in the same manner. Repeat the process until the growth from all tubes has been suspended.

If bouillon is used this should be centrifuged at high speed, the bouillon removed and the sediment containing the bacteria resuspended in salt solution. If the bouillon contains other than human serum it should be again centrifuged and resuspended in salt solution.

7. Transfer the suspension to a sterile flask or bottle containing sterile beads and shake well to break up clump (clumps of Staphylococci and typhoid bacilli are easily broken up; Streptococci, pneumococci and diphtheroids require more shaking).

8. Filter the suspension through sterile paper (it is convenient to have on hand small funnels with folded paper in place wrapped in newspaper and sterilized in hot air oven).

9. Estimate the number of bacteria per cubic centimeter in the suspension by one of the methods described on page 237.

10. After standardizing the vaccine, sterilize by heating it in a water bath 60° C. for 1 hour. This can readily be done by placing the suspension in a test tube or flask and placing in water bath in such a manner that the water is above the level of the suspension.

11. After heating, inoculate suitable media with either a dozen loopfuls or several drops of the vaccine. The cultures should be incubated for 24 hours, and if cultures are not sterile it should be heated again for another hour or, better, discarded and another prepared.

12. The number of organisms per cubic centimeter of vaccine to use should be determined. The following may be of aid:

Staphylococci	1,000,000,000
Streptococci	500,000,000
Pneumococci	500,000,000
Gonococci	500,000,000
Micrococcus catarrhalis	500,000,000
Bacillus typhosus	1,000,000,000
Bacillus paratyphosus	1,000,000,000
Bacillus coli	1,000,000,000
Bacillus pyocyaneus	1,000,000,000
Bacillus influenzæ	1,000,000,000

13. The vaccine should now be diluted with sterile salt solution, so each cubic centimeter will contain the desired number of bacteria.

14. Add a preservative to prevent chance contamination. The ones commonly used are phenol, 0.25 to 0.5 per cent, or tricresol, 0.2 to 0.5 per cent.

15. Place vaccine in sterile vaccine bottle or in ampules and properly label.

Methods for Standardizing Vaccines

By the Method of Wright

1. Make a mark on the stem of a capillary pipet about one inch from the tip and fit a rubber bulb to its barrel.

2. Cleanse and prick the finger.

3. Draw up into the capillary pipet sodium citrate solution to the mark on the tip. Then draw a little air in, then blood from the finger up to the mark. Draw a little air in to separate the solution, next draw bacterial suspension up to the mark.

4. Expel the contents of the pipet on a glass slide or in a watch glass and mix thoroughly by aspirating and re-expelling about a dozen times.

5. Make two or three thin films on slides in the same manner as described for blood smears (see page 17).

6. Dry in air and fix with a saturated solution of corrosive sublimate.

7. Wash and stain with dilute carbolfuchsin (1:10) or carbolthionin for two to five minutes.

8. Wash and dry.

9. Examine only satisfactory films which show bacteria and blood cells in approximately the same numbers and free from bacterial aggregates. With oil immersion lens count the number of corpuscles and bacteria in a number of fields, or until 500 corpuscles have been counted. Mark down the number of cells and bacteria counted separately and total each at the end.

Calculation:—Let us assume that 500 red cells and 1000 bacteria have been counted. A cubic millimeter of blood contains 5,000,000 red corpuscles and equal volumes of blood and emulsion were taken. A cubic millimeter of the emulsion, therefore, contains $\frac{5{,}000{,}000 \times 1000}{500} = 10{,}000{,}000$ organisms per cubic millimeter or 10,000,000,000 per cubic centimeter.

By the Nephelometer Method (McFarland)

1. Place 1 c.c. of bacterial suspension in test tube which should be of the same size as those used in the nephelometer.

2. Dilute with 4 to 10 c.c. of salt solution, keeping accurate record of the final dilution.

3. Compare with tubes of nephelometer. Shake the tubes well before comparing.

Calculation:—Multiply the number of bacteria represented by the nephelometer tube which corresponds with the density of the bacterial suspension by the dilution of the bacterial suspension. For example, assume that the density of the bacterial suspension corresponds to the No. 3 tube of the nephelometer and before making comparison it was diluted 8 times. Number 3 tube corresponds to 1,000,000,000; eight times this number equals 8,000,000,000 bacteria per cubic centimeter.

Preparation of Nephelometer

1. Arrange 10 test tubes or ampules of uniform size in rack, and label 1 to 10.

2. Add the following amount of a 1:40 solution of chemically pure barium chlorid: to tube No. 1, 0.1 c.c.; tube No. 2, 0.2 c.c.; and so on, increasing 0.1 c.c. in each.

3. Add sufficient of a 1 per cent chemically pure sulphuric acid solution to make the total volume in each tube 10 c.c.

4. Seal the tubes or ampules.

5. When the fine white precipitate of barium sulphate which has formed in the tubes is shaken up well, each tube will have a different density which increases from No. 1 to 10. The density of the tubes corresponds to bacterial suspensions as follows:

Tube	1	equals	300,000,000
Tube	2	"	600,000,000
Tube	3	"	900,000,000
Tube	4	"	1,200,000,000
Tube	5	"	1,500,000,000
Tube	6	"	1,800,000,000
Tube	7	"	2,100,000,000
Tube	8	"	2,400,000,000
Tube	9	"	2,700,000,000
Tube	10	"	3,000,000,000

6. This method is most suitable for vaccines prepared from solid agar slants as Staphylococcus and typhoid vaccines; it is not suitable for suspensions colored with the culture medium.

SECTION III

SEROLOGY

SECTION III

CLINICAL SEROLOGICAL METHODS

Methods for Collecting Blood

From a Vein

1. For the Wassermann test, blood should not be taken immediately after a meal (is likely to be chylous and serum anticomplementary) nor during or immediately after an acute alcoholic debauch.

2. Place a tourniquet about the arm above the elbow; request patient to vigorously open and close the hand.

3. Cleanse the skin over a prominent vein with tincture of iodin and alcohol.

4. Remove 5 to 10 c.c. of blood with a sterilized Luer or Record syringe fitted with a No. 18 needle. Transfer the blood to a small sterile test tube, vial or other suitable container.

5. The Keidel tube is particularly convenient. A modified Keidel tube made by Harris Comer, Philadelphia, enables one to see a flow of blood and, therefore, that the vein has been entered, before the neck of the vial is broken. This tube is particularly convenient. After filling with blood the ampule should be sealed by bending over the rubber stem with a rubber band to prevent loss if specimen is mailed.

6. Release the tourniquet and then withdraw the needle.

7. As a general rule the wound requires no further attention unless several punctures have been made, in which case it should be cleansed with alcohol and covered with flexible collodion.

From a Finger

1. Small amounts of blood for agglutination, Wassermann, transfusion and other tests are easily obtained by puncturing a finger.

2. The hand must be warm with good circulation. If cold and clammy have patient immerse the hand for a few minutes in hot water and rub briskly with a towel.

3. Cleanse tip of finger and Daland lancet (broad blade) with alcohol; dry.

4. Puncture across the lines at tip of finger.

5. Massage blood into a small test tube; with a large tube too much is lost on the sides.

Method for Separating the Serum

1. If serum is desired at once allow the blood to coagulate for a few minutes, break up thoroughly with a rod and centrifuge.

2. If serum is not needed at once, place specimen in a refrigerator for spontaneous contraction of the clot and separation of serum. If this is unsatisfactory break up the clot with a rod and centrifuge.

Method for Preservation of Serum

1. Sterile serum may be kept in sterile vials or other containers in a refrigerator without a preservative.

2. Otherwise it is well to add a preservative, as 0.25 per cent phenol. Keep a 5 per cent stock solution and add 0.05 c.c. to each cubic centimeter of serum.

3. Rabbit serum hemolysins may be preserved by adding to the serum an equal part of chemically pure glycerin; mix well and keep in refrigerator. Hemolytic sera may be also dried on filter paper (Noguchi) and kept in refrigerator.

4. Immune sera (antitoxins, agglutinins) may be preserved in dried powder form.

5. Guinea pig complement serum may be preserved for at least three weeks (sometimes longer) by adding 0.1 gm. of pure sodium chlorid to each cubic centimeter and keeping on ice. For use dilute 1 c.c. with 9 c.c. of water which gives a 1:10 isotonic solution. For higher dilutions dilute with saline (for 1:30 dilute 1 part of 1:10 with 2 parts of saline). Preserved complement loses first in fixability by antigen and antibody in syphilis before loss in hemolytic activity.

Technic of Microscopical Agglutination Tests

Widal Test with Dried Blood

1. Puncture finger or lobe of ear and collect a few drops of blood on a clean glass slide or paper and allow it to dry (do not heat to hasten drying).

2. Place a loopful of a 24 hour culture in the center of a clean cover glass.

3. Moisten the dried blood with a loopful of normal salt solution. Rub up the dried blood and transfer sufficient to the cover glass and mix thoroughly with the drop of culture. The mixture should have a delicate orange-pink color.

4. Mount the cover glass with vaselin on a concave slide, and allow it to stand one hour.

5. Prepare another slide in the same manner using the culture only (control).

6. Examine both slides with the 1/6 objective.

A positive reaction is indicated by clumping of the bacilli and more or less loss of motility as compared with the control.

Widal Test with Serum

1. Prick finger and obtain 0.5 to 1.0 c.c. of blood or fill a Wright's capsule.

2. Draw off serum with capillary pipet after standing or centrifuging (serum should be clear and free of corpuscles).

3. Take two small watch crystals or hollow slides and place one drop of serum in each.

4. Add to one 24 drops of normal salt solution and to the other 49 drops, making dilutions of 1 : 25 and 1 : 50, respectively.

5. Place one loopful of an 18 to 24 hour bouillon culture of typhoid bacilli in the middle of each of three cover glasses.

6. To the first, mix one loopful of serum diluted 1 : 25.
   To the second, mix one loopful of serum diluted 1 : 50.
   To the third, mix one loopful of normal salt solution.

7. Mount each in vaselin on hanging drop slide. The final dilutions obtained are 1 : 50 and 1 : 100; therefore mark the slides as follows: No. 1, 1 : 50; No. 2, 1 : 100; and No. 3, control.

8. Allow to stand at room temperature for one hour.

9. Examine with 1/6 objective. Control should be inspected first and should not show any clumping or loss of motility.

10. Examine the 1 : 50 and 1 : 100 dilutions for clumping and loss of motility.

Macroscopical Agglutination Tests

For Typhoid and Paratyphoid Fevers

1. *Antigen.*—Prepare suitable suspension of the organism to be used in the test. For typhoid, paratyphoid and other intestinal organisms the method of Dreyer is recommended.

(*a*) Inoculate flask of bouillon with a good growing strain of the organism.

(*b*) At the end of 24 to 48 hours, if good growth is obtained, shake well and add sufficient formalin to make 1.0 per cent (10 c.c. commercial formalin to 1000 c.c. of culture).

(*c*) Shake well and place in ice box at 2° C.

(*d*) Shake daily and make subcultures for sterility.

(*e*) At the end of 3 or 4 days the culture should be found perfectly sterile. It is then transferred to sterile bottles with rubber stoppers and kept in cold dark place.

2. If this antigen is not available a good suspension may be prepared by washing off agar slant cultures with phenolized saline solution (0.5 per cent in saline); shake with glass beads and filter through sterile paper. The suspension should be heavy enough for the tests; each cubic centimeter should contain at least 2,000,000,000 bacilli. Or a good 48 to 72 hour broth culture may be employed after heating for an hour in a water bath at 60° C.

3. Secure at least 1 c.c. of blood by finger puncture.

4. Place seven test tubes in rack and add 1 c.c. of sterile distilled water to each. Mark tubes 1 to 7.

5. Separate the serum from the clot and make a 1 : 5 dilution (0.2 c.c. serum to 0.8 c.c. of water).

6. Add 1 c.c. of the diluted serum (1 : 5) to the first tube and mix thoroughly.

7. Place 1 c.c. of serum from tube No. 1 into tube No. 2 and mix thoroughly; then place 1 c.c. from tube No. 2 into tube No. 3 and so on until the sixth tube is reached; then discard 1 c.c. from this tube, as no serum is to be added to the seventh tube which is the culture control tube.

8. Add 1 c.c. of the bacterial suspension (antigen) to each tube, which doubles the serum dilutions. The tubes will now contain the following dilutions of serum:

No. 1	equals	1:20
No. 2	"	1:40
No. 3	"	1:80
No. 4	"	1:160
No. 5	"	1:320
No. 6	"	1:640
No. 7	"	None (control)

9. Mark the dilution on each tube with wax pencil. Shake gently, plug with cotton and place in the incubator for 2 hours. The tubes are then allowed to stand at room temperature for a few hours or in the refrigerator over night, after which readings are made.

10. Inspect the control tube first; it should show uniform cloudiness.

A positive reaction shows masses or clumps of bacteria adhering to the sides and bottom of the tube which are broken with difficulty, and the supernatant fluid is clear.

11. If the tests are conducted with the serum of an individual vaccinated with typhoid-paratyphoid vaccine and suspected as having typhoid fever, the test must be done every two or three days with the *same antigen*, and great technical care. A definite rise in agglutination titer indicates typhoid infection; if the titer remains practically unchanged typhoid very probably is not present.

Macroscopic Slide Widal Test (Bass and Johns)

1. Prepare a suspension of typhoid bacilli containing 10,000,000 per c.c.; heat in a water bath at 60° C. for 1 hour and preserve with 1 per cent formalin (to each 99 c.c. add 1 c.c. of commercial formalin). Keeps 6 to 12 months or longer. Shake before using.

2. Prepare a film of blood on a slide in the usual manner, using about ¼ drop.

3. After drying, place 1 drop of water on the film and dissolve the blood with a tooth pick or other suitable instrument.

4. Add 1 drop of the suspension of typhoid bacilli and mix with the blood.

5. A positive reaction occurs in 2 minutes, with the formation of small grayish clumps and fine granular sediment. Do not mistake dust particles for agglutination.

Macroscopic Slide Method for Plate Cultures

1. This test is for the rapid identification of bacteria as typhoid, paratyphoid and dysentery bacilli in colonies on plates of feces and meningococci, in colonies on plates of cultures of the nasopharynx. A good potent immune serum must be available; the titer must be known in order to avoid group reactions or falsely negative results.

2. On a glass slide place a drop of diluted immune serum; a drop of saline is placed on the other end or on a second slide.

3. With a sterilized wire pick up a portion of the suspicious colony and emulsify in the drop of saline (control); sterilize and pick up portion and emulsify in the serum.

4. In a positive reaction clumping occurs almost at once; spontaneous agglutination is detected in the control.

Macroscopic Absorption Test for Differentiating between Typhoid and Paratyphoid Fevers

1. Arrange four rows of four small test tubes, each row to contain 1 c.c. of serum dilutions 1 : 20, 1 : 40, 1 : 80 and 1 : 160, respectively.

2. In each tube of the first and second rows emulsify five large loopfuls of typhoid bacilli. Add an additional control tube carrying 1 c.c. of saline and culture.

3. In each tube of the third and fourth rows place paratyphoid bacilli (A or B as decided); add a control tube.

4. Mix gently and water bath at 37° C. for two hours. Record results.

5. Centrifuge all tubes except controls and transfer supernatant fluids to four more rows of tubes.

6. To each tube of the first and third rows add typhoid bacilli; to the second and fourth rows add paratyphoid bacilli. Mix well and place in water bath two hours at 38° C.

7. If typhoid fever is present, agglutination will be strong in the first and second rows of the first part of the test and practically unchanged in the third row of the second part.

8. If paratyphoid is present agglutination will be strong in

the third and fourth rows in first part of the test and practically unchanged in the fourth row of the second part. If paratyphoid B is employed with negative results, repeat the test with paratyphoid A.

Method for Typing Pneumococci

1. Wash a bean sized portion of sputum with sterile salt solution and emulsify with a little salt solution in a sterile mortar.
2. Inoculate the material into the peritoneal cavity of a mouse.
3. At the end of the eight to ten hours remove a drop of peritoneal exudate with a sterile capillary pipet and examine for pneumococci.
4. If pneumococci are abundant kill the mouse and wash out the peritoneal cavity with 4 or 5 c.c. of normal salt solution and collect the washings in a centrifuge tube.
5. Centrifuge at low speed for 2 or 3 minutes (this will throw down the endothelial and pus cells leaving the organisms in suspension).
6. Remove the supernatant fluid, place it in another tube, and centrifuge at high speed.
7. Remove the supernatant fluid and emulsify the sediment with normal salt solution making a moderately heavy suspension (should equal at least an 18 hour bouillon culture of pneumococcus).
8. Place 0.5 c.c. of this emulsion into each of six small test tubes.
9. Into the first tube add 0.5 c.c. of pneumococcus serum, type I (undiluted); to the second tube add type II serum (undiluted); to the third tube add type II serum (diluted 1 : 20); to the fourth tube add type III serum (undiluted); to the fifth tube add a few drops of bile to determine if the emulsion contains chiefly bile soluble organisms. To the sixth tube add 0.5 c.c. saline (control).
10. Mix well; water bath at 37° C. for one hour.
11. Agglutination is indicated by a flocculent appearance in contrast to uniform cloudiness of the control, along with rapid settling of the agglutinated organisms leaving the fluid above clear. The bile tube clears; if not, the suspension is probably not of pneumococci.
12. If agglutination takes place in the tube containing type I

serum and not in any of the other tubes the strain is type I; if agglutination occurs in both the tubes containing type II serum (undiluted and diluted) the strain is a typical type II; if agglutination occurs only in the tube containing undiluted type II serum the strain is an atypical type II; if agglutination occurs in the tube containing type III serum the strain is type III. If no agglutination takes place in any of the tubes the strain belongs to type IV.

Method for Typing Meningococci

1. Have on hand the different type immune sera.
2. Prepare a suspension of the meningococcus in saline (at least 2,000,000,000 per c.c.); heat at 65° C. in water bath for thirty minutes and add 0.5 per cent phenol (1 c.c. of 5 per cent for each 9 c.c. of suspension).
3. Arrange four dilutions of each immune serum in 1 c.c. amounts 1 : 25, 1 : 50, 1 : 100, 1 : 200. To each tube and a control carrying 1 c.c. of saline, add 1 c.c. of suspension.
4. Mix well; incubate at 55° C. for sixteen hours.
5. The control should show no agglutination; inspect other tubes for presence of agglutination which indicates the type of meningococcus according to type serum producing the reaction.

Methods for Blood Transfusion Tests

Direct Matching Microscopical Test for Agglutination of Corpuscles

1. Prepare finger of recipient and make deep puncture.
2. Collect 10 to 15 drops of blood in a small test tube containing 1 c.c. of 2 per cent sodium citrate solution.
3. At the same time secure 1 to 2 c.c. of blood in dry test tube.
4. Secure blood from the donor in the same manner.
5. Break up the clots and centrifuge along with the tubes containing the citrated blood.
6. Remove the supernatant fluids from the citrated tubes and add to the corpuscles in each tube 2 volumes of normal salt solution; suspend the corpuscles.
7. Hanging drop preparations are prepared as follows:

   (*a*) 2 loopfuls of patient's serum plus 1 loopful of donor's corpuscles are mixed on cover glass and mounted with vaselin on hanging drop slide.

(*b*) 2 loopfuls of donor's serum plus 1 loopful of patient's corpuscles are mixed and mounted.

(*c*) 2 loopfuls of saline and 1 loopful of patient's corpuscles are mixed and mounted (control).

(*d*) 2 loopfuls of saline plus 1 loopful of donor's corpuscles are mixed and mounted (control).

8. Allow to stand for 15 minutes at room temperature.

9. Examine with 2/3 objective. Controls should be examined first (*c* and *d*) and should not show any agglutination. False clumping and rouleaux formation at the margin of the mixture should not be mistaken for a positive reaction.

10. Agglutination in *a* shows that the patient's serum is incompatible with the donor's corpuscles. Agglutination in *b* shows that the donor's serum is incompatible with the patient's corpuscles.

11. Repeat the test with the corpuscles and serum of each donor.

12. This direct test is always advisable before transfusion even if the patient is first grouped and a donor of the same group is selected in order to guard against the possibility of transfusing with an incompatible subgroup blood.

Direct Macroscopical Test for Agglutination and Hemolysis of Corpuscles

1. Collect blood in citrate solution and in a small dry test tube from the patient and donor as just described.

2. Prepare the corpuscles and secure the sera as described.

3. Arrange 4 small test tubes and place 1 c.c. of saline in each.

4. Into No. 1 place 5 drops of patient's serum and 1 drop of donor's corpuscles.

5. Into No. 2 place 5 drops of donor's serum and 1 drop of patient's corpuscles.

6. Into No. 3 place 1 drop of patient's corpuscles (control).

7. Into No. 4 place 1 drop of donor's corpuscles ((control).

8. Mix each tube and place in a water bath at 37° C. for one hour.

9. Read the results. Tubes 3 and 4 should show no hemolysis or agglutination.

10. If hemolysis and agglutination do not occur in tubes 1 and 2 the bloods are compatible. If the patient's serum is incompatible

with the donor's corpuscles, a positive reaction will occur in tube No. 1; if the donor's serum is incompatible with the patient's corpuscles a positive reaction will occur in tube No. 2.

11. Repeat in same manner with the serum and corpuscles of each donor.

Microscopical Blood Grouping Test

1. Prepare finger and make puncture of the individual to be grouped.

2. Collect 2 or 3 drops of blood in small test tube containing 0.5 to 1.0 c.c. of a 1 per cent sodium citrate in normal salt solution.

3. Test the cells thus obtained with known group II and group III sera (Moss) by examination of the following hanging drop preparations:

(*a*) 2 loopfuls of group II serum plus 1 loopful of corpuscle suspension are mixed on a cover glass and mounted with vaselin on a hanging drop slide.

(*b*) 2 loopfuls of group III serum plus 1 loopful of corpuscle suspension are mixed and mounted.

(*c*) 2 loopfuls of normal saline plus 1 loopful of corpuscle suspension are mixed and mounted (control).

4. Allow to stand for 15 minutes and examine with 2/3 objective.

5. The group is determined as follows:

No agglutination with either group II or III serum = group I of Jansky or group IV of Moss.

Agglutination with both groups II and III serum = group IV of Jansky or group I of Moss.

Agglutination with group II but no agglutination with group III serum = group III of both Jansky and Moss.

Agglutination with group III but not with group II serum = group II of both Jansky and Moss.

6. False clumping and rouleaux formation at the margin of the mixture should not be mistaken for a positive reaction.

7. Known group II and group III sera should be kept in a refrigerator preserved with 0.25 per cent phenol. It is well to replenish every 2 or 3 months and frequent tests should be made to make sure that the sera are potent. It is easy to replenish the

sera by typing the corpuscles of blood specimens submitted for the Wassermann tests. If corpuscles belonging to group II are found, the excess serum may be preserved (group II); if corpuscles belonging to group III are found, the excess serum should be preserved (group III). These two sera suffice for grouping by either the Jansky or Moss classifications. Group I and group IV sera are unnecessary.

Technic of the New Kolmer Quantitative Complement Fixation Test for Syphilis

Special Glassware and Apparatus Required

(*a*) Pipets: 1 c.c. graduated in 0.01 c.c. to tip.
5 c.c. " " 0.10 c.c.
10 c.c. " " 0.50 c.c.
14.4 c.c. " " 1.20 c.c.

(*b*) Test tubes: 85 mm. × 13 mm. (inside diameter) with rounded bottom and no lips.

(*c*) Cylinders: glass stoppered, graduated (50 or 100 c.c. capacity) to be used for measuring amounts over 50 c.c.

(*d*) Centrifuge tubes: special graduated Kolmer centrifuge tubes of two sizes, 10 c.c. and 30 c.c. capacity.

(*e*) Test tube racks: galvanized wire racks carrying 12 rows of 6 tubes each.

(*f*) Water bath: any easily regulated water bath is suitable, or a simple galvanized pan carrying water to a depth of 8 cm. can be used satisfactorily at 55° C. or 37° C.

(*g*) Refrigerator: any refrigerator maintaining a temperature of 6° to 8° C. is satisfactory.

Method of Cleaning Glassware

(*a*) All glassware should be chemically clean and preferably sterile. To clean tubes and flasks empty and rinse in running tap water; wash inside and outside in soapy water; rinse several times in running tap water and invert in wire baskets. Dry in the hot air oven at about 160° C.

(*b*) Pipets should be placed after use in a jar or cylinder of clean water with a pad of cotton in the bottom. To clean pipets

rinse thoroughly in running tap water, place in a metal box or wire basket and sterilize in the oven.

(*c*) Flasks should be plugged with cotton and sterilized in the oven until the cotton turns a light brown.

(*d*) If glassware becomes cloudy, immerse in bichromate cleaning fluid (2 parts potassium bichromate, 3 parts commercial sulphuric acid, and 25 parts water) for 24 hours. Rinse thoroughly in running tap water and proceed with the washing as before.

Reagents

(*a*) *Physiological Salt Solution.*—Used as a diluting fluid. Dissolve 8.5 grams of dry, chemically pure sodium chlorid in 1000 c.c. of distilled water. If the salt has absorbed moisture it should be dried in the hot air oven for 10 or 15 minutes before weighing. Filter solution through paper into a flask fitted with a gauze-covered cotton stopper. Sterilize by heating in an Arnold sterilizer for one hour.

(*b*) *Sheep Corpuscles* (*Indicator Antigen*).—Sheep blood may be obtained at an abattoir or by bleeding a sheep from the external jugular vein. Collect the blood in a sterile glass jar containing glass beads and shake gently for 5 or 10 minutes to defibrinate. Keep the defibrinated blood in the refrigerator at as low a temperature as possible. Blood should keep for a week without preservative or for 2 or 3 weeks if formalin is added in sufficient quantity to make a dilution of 1 : 800.

Filter a small quantity of blood through cotton into a graduated centrifuge tube. Allow twice as much blood as the amount of cells required. Add 2 or 3 volumes of physiological salt solution. Centrifuge at moderate speed until all the corpuscles are thrown down.

Remove the supernatant fluid with a capillary pipet. Add 3 or 4 volumes of salt solution; mix by inverting and centrifuge again for the same length of time.

Repeat the process for a third time but centrifuge twice as long as in the first washing in order to pack the cells evenly and firmly.

Cells should be washed until the supernatant fluid is colorless. Three washings are usually sufficient. (If more than four washings are necessary the cells are too fragile for use).

Read the volume of cells in the centrifuge tube, carefully re-

move the supernatant fluid and prepare a 2 per cent suspension by washing the corpuscles into a flask with 49 volumes of physiological salt solution. Always shake before using to secure an even suspension, as the corpuscles settle to the bottom of the flask when not in use.

(*c*) *Antisheep Hemolysin.*—Give a rabbit 5 or 6 intravenous injections of 5 c.c. of a 10 per cent suspension of washed sheep corpuscles every 4 or 5 days. Bleed the rabbit 7 to 9 days after the last injection if a preliminary titration gives a unit of 0.5 c.c. of 1 : 4000 or higher. Separate the serum, heat at 55° C. for ½ hour and preserve with an equal part of best grade neutral glycerin.

(*d*) *Complement.*—The pooled sera of at least 3 healthy guinea pigs should be used. Select large, well-nourished animals that have not been fed for 12 hours; avoid pregnant animals.

Anesthetize the pig lightly with ether. Sever the large blood vessels on both sides of the neck, being careful not to cut the esophagus. Collect the blood in a centrifuge tube by means of a large funnel. Place the blood in an incubator at 38° C. for one hour; break up the clot and centrifuge. Separate the clear serum. Keep in the refrigerator when not in use.

If but a small amount of serum is required 4 or 5 c.c. of blood may be obtained from each pig by bleeding from the heart with a 5 c.c. syringe fitted with a short needle, gauge 20.

Complement serum may be preserved for several weeks by adding 0.3 gm. of C.P. sodium chlorid to each cubic centimeter of serum. Keep in a dark glass bottle at or near the freezing point. To prepare for use dilute 1 c.c. of serum with 29 c.c. of distilled water. This gives a 1 : 30 dilution in 1 per cent salt solution. Or the serum may be preserved with 0.1 gm. NaCl per c.c. and diluted 1 : 10 with 9 c.c. of water; for 1 : 30 dilute with 2 parts of saline.

(*e*) *Antigen.*—A new cholesterolized and lecithinized alcoholic extract of heart muscle is employed.

Bacto-Beef Heart, prepared by the Digestive Ferments Company of Detroit, is recommended.

Place 25 gm. of powder and 200 c.c. of ether in a tightly stoppered bottle. Extract at room temperature for 5 days, shaking occasionally each day. This extraction may also be made with 100 c.c. of ether in a Soxhlet for 18 hours.

Filter off the extract and keep in a tightly stoppered bottle. This

is the primary ether extract. Dry the powder by spreading on a glass plate. Return to bottle and extract with 200 c.c. of 95 per cent alcohol in the incubator for 4 days, shaking occasionally.

Filter off the alcoholic extract, place in a shallow dish and evaporate to dryness by fanning, or in the incubator. Dissolve the residue in 30 to 50 c.c. of ether. Cover and let stand for an hour or two. Pour off the clear ether extract and to it add the primary ether extract. Concentrate the mixture by fanning until it is reduced to about one-fourth of its volume. Add six volumes of pure acetone. Cover and set aside for several hours to allow the precipitate to settle. Decant the supernatant acetone and place the sticky residue in a wide-mouthed bottle. Cover with acetone and keep for future use. This is the acetone insoluble lipoid portion.

To the muscle powder after this extraction add 100 c.c. of absolute acetone-free ethyl alcohol. Extract in the incubator for 6 days, shaking occasionally, and if possible shake in a mechanical shaker for 1 day. Filter through fat-free paper.

To the acetone insoluble lipoids add 0.2 gm. of pure cholesterol. Dissolve the mixture in 10 c.c. of pure ether; add the cloudy brownish mixture slowly to the filtered alcoholic extract and shake well.

Place the antigen in the incubator over night; then keep at room temperature for 1 or 2 days, shaking occasionally. Filter through fat-free paper. Keep in a tightly stoppered brown glass bottle at room temperature or in the refrigerator.

Titration of Hemolysin and Complement

These titrations must be made each time the complement fixation test is conducted.

For the *hemolysin titration* prepare a stock dilution of 1 : 100 hemolysin as follows:

Glycerinized hemolysin	2.0 c.c.
Physiological salt solution	94.0 c.c.
5 per cent phenol in salt solution	4.0 c.c.

This may be kept in the refrigerator for several weeks.

For titration dilute to 1 : 1000 (0.5 c.c. of 1 : 100 + 4.5 c.c. salt solution).

In a series of 10 tubes prepare dilutions as follows:

1. 0.5 c.c. of 1 : 1000 = 1 : 1000
2. 0.5 c.c. of 1 : 1000 + 0.5 c.c. salt solution = 1 : 2000
3. 0.5 c.c. of 1 : 1000 + 1.0 c.c. " " = 1 : 3000
4. 0.5 c.c. of 1 : 1000 + 1.5 c.c. " " = 1 : 4000
5. 0.5 c.c. of 1 : 1000 + 2.0 c.c. " " = 1 : 5000
6. 0.5 c.c. of 1 : 3000 + 0.5 c.c. " " = 1 : 6000
7. 0.5 c.c. of 1 : 4000 + 0.5 c.c. " " = 1 : 8000
8. 0.5 c.c. of 1 : 5000 + 0.5 c.c. " " = 1 : 10000
9. 0.5 c.c. of 1 : 6000 + 0.5 c.c. " " = 1 : 12000
10. 0.5 c.c. of 1 : 8000 + 0.5 c.c. " " = 1 : 16000

Mix the contents of each tube thoroughly.

Prepare 1 : 30 dilution of complement for hemolysin and complement titrations by diluting 0.2 c.c. of complement serum with 5.8 c.c. salt solution.

Prepare a 2 per cent suspension of sheep corpuscles.

In a series of 10 tubes set up the hemolysin titrations as follows:

TABLE VIII

Tube	Hemolysin	Complement 1 : 30	Physiological Salt Solution	Corpuscles
1	0.5 c.c. of 1 : 1000	0.3 c.c.	1.7 c.c.	0.5 c.c.
2	" " 1 : 2000	"	"	"
3	" " 1 : 3000	"	"	"
4	" " 1 : 4000	"	"	"
5	" " 1 : 5000	"	"	"
6	" " 1 : 6000	"	"	"
7	" " 1 : 8000	"	"	"
8	" " 1 : 10000	"	"	"
9	" " 1 : 12000	"	"	"
10	" " 1 : 16000	"	"	"

Mix the contents of each tube and incubate in the water bath at 38° C. for one hour. Read the unit of hemolysin. *The unit is the highest dilution of hemolysin that gives complete hemolysis.*

Two units are used in the complement and antigen titrations and in the complement fixation tests. Hemolysin is so diluted

that 0.5 c.c. contain 2 units. For example, if the unit equals 0.5 c.c. of 1 : 6000, 2 units equal 0.5 c.c. of 1 : 3000. Dilute just enough hemolysin for the complement titration and the complement fixation tests. Keep hemolysin and corpuscles in suspension in the refrigerator when not in use.

For the complement titration use 1 : 30 dilution of complement prepared above. Dilute antigen so that 10 units are contained in 0.5 c.c. (see antigen titration, page 275). This dilution is made by placing the required amount of physiological salt solution in a flask and adding antigen drop by drop. Shake the flask after each addition of antigen. Prepare enough antigen dilution for the complement titration and the complement fixation tests.

In a series of 10 test tubes set up the complement titrations as follows:

TABLE IX

Tube	Complement 1 : 30	Antigen 10 units	Physiological Salt Solution	WATER BATH 38° C. FOR ONE HOUR	Hemolysin 2 units	Corpuscles 2 per cent	WATER BATH 38° C. FOR ONE HOUR
1	0.1 c.c.	0.5 c.c.	1.4 c.c.		0.5 c.c.	0.5 c.c.	
2	0.15 c.c.	"	1.4 c.c.		"	"	
3	0.2 c.c.	"	1.3 c.c.		"	"	
4	0.25 c.c.	"	1.3 c.c.		"	"	
5	0.3 c.c.	"	1.2 c.c.		"	"	
6	0.35 c.c.	"	1.2 c.c.		"	"	
7	0.4 c.c.	"	1.1 c.c.		"	"	
8	0.45 c.c.	"	1.1 c.c.		"	"	
9	0.5 c.c.	"	1.0 c.c.		"	"	
10	—	—	2.5 c.c.		—	—	

The smallest amount of complement giving complete hemolysis is the *exact* unit; the next higher tube is the *full* unit. *Two full* units of complement (the dose) are used in the test and are so diluted that 1 c.c. contains the dose.

Example: Exact unit = 0.25 c.c. of 1 : 30 dilution
Full unit = 0.30 c.c. of 1 : 30 dilution
2 full units = 0.60 c.c. of 1 : 30 dilution

To calculate the dilution to use so that 1 c.c. contains the dose, divide 30 by the dose:

$$30 \div 0.6 = 50 \text{ or dilution } 1:50$$

Titration of Antigen

The antigen is titrated for the hemolytic, anticomplementary and antigenic units.

For the hemolytic and anticomplementary titrations prepare the following dilutions:

0.5 c.c.	Antigen		+ 1.5 c.c.	salt solution	= 1:4
0.5 c.c.	"		+ 2.0 c.c.	" "	= 1:5
0.5 c.c.	"		+ 2.5 c.c.	" "	= 1:6
1.0 c.c.	"	(1:4)	+ 1.0 c.c.	" "	= 1:8
1.5 c.c.	"	(1:5)	+ 1.5 c.c.	" "	= 1:10
1.0 c.c.	"	(1:6)	+ 1.0 c.c.	" "	= 1:12
1.0 c.c.	"	(1:8)	+ 1.0 c.c.	" "	= 1:16
1.0 c.c.	"	(1:10)	+ 1.0 c.c.	" "	= 1:20
1.0 c.c.	"	(1:12)	+ 1.0 c.c.	" "	= 1:24
1.0 c.c.	"	(1:16)	+ 1.0 c.c.	" "	= 1:32

Prepare a 1:10 dilution of *normal negative* human serum inactivated at 55° C. for 15 minutes.

For the *hemolytic titration* set up a series of 10 tubes as follows:

TABLE X

Tube	Antigen	Normal Human Serum 1:10	Physiological Salt Solution	SHAKE TUBES GENTLY AND PLACE IN REFRIGERATOR AT 6° TO 8° C. FOR 16 TO 18 HOURS.	Corpuscles 2 per cent	WATER BATH 38° C. FOR ONE HOUR
1	0.5 c.c. of 1:4	0.5 c.c.	1.5 c.c.		0.5 c.c.	
2	" " 1:5	"	"		"	
3	" " 1:6	"	"		"	
4	" " 1:8	"	"		"	
5	" " 1:10	"	"		"	
6	" " 1:12	"	"		"	
7	" " 1:16	"	"		"	
8	" " 1:20	"	"		"	
9	" " 1:24	"	"		"	
10	" " 1:32	"	"		"	

After secondary incubation place tubes in the refrigerator for several hours and read the results. The *hemolytic unit* is the smallest amount of antigen just beginning to produce hemolysis. As a rule this antigen is not hemolytic in dilutions higher than 1 : 4.

For the *anticomplementary titration* set up a series of 12 tubes as follows:

TABLE X

Tube	Antigen	Normal Human Serum 1 : 10	Complement 2 full units	SHAKE TUBES GENTLY AND PLACE IN REFRIGERATOR AT 6° TO 8° C. FOR 16 TO 18 HOURS, FOLLOWED BY WATER BATH AT 38° C. FOR 5 TO 10 MINUTES.	Hemolysin 2 units	Corpuscle 2 per cent	SHAKE AND PLACE IN WATER BATH AT 38° C. FOR 1 HOUR.
1	0.5 c.c. of 1 : 4	0.5 c.c.	1.0 c.c.		0.5 c.c.	0.5 c.c.	
2	" " 1 : 5	"	"		"	"	
3	" " 1 : 6	"	"		"	"	
4	" " 1 : 8	"	"		"	"	
5	" " 1 : 10	"	"		"	"	
6	" " 1 : 12	"	"		"	"	
7	" " 1 : 16	"	"		"	"	
8	" " 1 : 20	"	"		"	"	
9	" " 1 : 24	"	"		"	"	
10	" " 1 : 32	"	"		"	"	
11	Salt sol. 0.5 c.c.	"	"		"	"	
12	" " 1.0 c.c.	"	"		"	"	

Place tubes in the refrigerator for several hours and read. The *anticomplementary unit* is the smallest amount of antigen producing some inhibition of hemolysis. This antigen is usually anticomplementary in dilutions up to 1 : 6. Tube 11 is the serum control; tube 12 is the hemolytic system control. Both should show complete hemolysis.

For the *antigenic titration* proceed as follows: From the remainder of the 1 : 10 dilution of antigen prepared for the hemolytic and anticomplementary titrations make the following dilutions:

0.1 c.c.	antigen	1 : 10	to	2.9 c.c.	salt	solution	= 1 : 300
0.1 c.c.	"	1 : 10	"	3.9 c.c.	"	"	= 1 : 400
0.1 c.c.	"	1 : 10	"	4.9 c.c.	"	"	= 1 : 500
1.0 c.c.	"	1 : 300	"	1.0 c.c.	"	"	= 1 : 600
1.0 c.c.	"	1 : 400	"	1.0 c.c.	"	"	= 1 : 800
1.0 c.c.	"	1 : 500	"	1.0 c.c.	"	"	= 1 : 1000
1.0 c.c.	"	1 : 600	"	1.0 c.c.	"	"	= 1 : 1200
1.0 c.c.	"	1 : 800	"	1.0 c.c.	"	"	= 1 : 1600
1.0 c.c.	"	1 : 1000	"	1.0 c.c.	"	"	= 1 : 2000
1.0 c.c.	"	1 : 1200	"	1.0 c.c.	"	"	= 1 : 2400
1.0 c.c.	"	1 : 1600	"	1.0 c.c.	"	"	= 1 : 3200
1.0 c.c.	"	1 : 2000	"	1.0 c.c.	"	"	= 1 : 4000

Prepare a 1 : 10 dilution of a mixture of 6 or more strongly positive syphilitic sera heated at 55° C. for 15 minutes. Set up the titration as follows:

TABLE XI

Tube	Antigen	Positive Serum 1 : 10	Complement 2 full units	SHAKE TUBES GENTLY AND PLACE IN REFRIGERATOR AT 6° TO 8° C. FOR 16 TO 18 HOURS, FOLLOWED BY WATER BATH AT 38° C. FOR 5 TO 10 MINUTES.	Hemolysin	Corpuscles 2 per cent	SHAKE TUBES AND PLACE IN WATER BATH AT 38° C. FOR 1 HOUR.
1	0.5 c.c. of 1 : 300	0.5 c.c.	1 c.c.		0.5 c.c.	0.5 c.c.	
2	" " 1 : 400	"	"		"	"	
3	" " 1 : 500	"	"		"	"	
4	" " 1 : 600	"	"		"	"	
5	" " 1 : 800	"	"		"	"	
6	" " 1 : 1000	"	"		"	"	
7	" " 1 : 1200	"	"		"	"	
8	" " 1 : 1600	"	"		"	"	
9	" " 1 : 2000	"	"		"	"	
10	" " 1 : 2400	"	"		"	"	
11	" " 1 : 3200	"	"		"	"	
12	" " 1 : 4000	"	"		"	"	
13	Salt sol. 0.5 c.c.	"	"		"	"	
14	" " 1.0 c.c.	—	"		"	"	

Place tubes in refrigerator for several hours and read. The *antigenic unit* is the smallest amount of antigen producing complete inhibition of hemolysis. Tube 13 is the serum control; tube 14 is the hemolytic system control. Both should show complete hemolysis. As a general rule the unit is 0.5 c.c. of 1 : 2000 to 1 : 4000 dilutions.

In the complement fixation tests 10 antigenic units of antigen are used. The antigen is so diluted that 0.5 c.c. contains the dose. For example:

Antigenic unit = 0.5 c.c. of 1 : 2000
Dose (10 units) = 0.5 c.c. of 1 : 200

The antigen is satisfactory if the dose is 1/20 to 1/50 of the anticomplementary unit or less.

This antigen will keep for a year or longer with no change in antigenic or anticomplementary value.

The Quantitative Complement Fixation Test

(*a*) *For each serum* arrange 6 tubes and place in them the following amounts of salt solution, respectively: 1.2 c.c., 0.5 c.c., 0.5 c.c., 2.0 c.c., 0.5 c.c. and 0.5 c.c.

(*b*) To tube 1 add 0.3 c.c. of inactivated serum. Mix by drawing up in the pipet several times. Transfer 0.5 c.c. to tube 2 and 0.5 c.c. to tube 6.

(*c*) Mix tube 2 and transfer 0.5 c.c. to tube 3.
Mix tube 3 and transfer 0.5 c.c. to tube 4.
Mix tube 4 and transfer 0.5 c.c. to tube 5; discard 1.5 c.c.
Mix tube 5 and discard 0.5 c.c.
Tubes 1 to 5 now contain 0.5 c.c., carrying 0.1, 0.05, 0.025, 0.005 and 0.0025 c.c. of serum. Tube 6 (serum control) contains 1.0 c.c. carrying 0.1 c.c. serum.

(*d*) *For each spinal fluid* arrange 6 tubes and place 0.5 c.c. salt solution in tubes 2, 3, 4, 5, and 6.
In tubes 1, 2 and 6 place 0.5 c.c. spinal fluid.
Mix tube 2 and transfer 0.5 c.c. to tube 3.
Mix tube 3 and transfer 0.5 c.c. to tube 4.
Mix tube 4 and transfer 0.5 c.c. to tube 5.
Mix tube 5 and discard 0.5 c.c.

Tubes 1 to 5 now contain 0.5 c.c. carrying 0.5, 0.25, 0.125, 0.0625 and 0.03125 c.c. of spinal fluid. Tube 6 (spinal fluid control) contains 1 c.c. carrying 0.5 c.c. of spinal fluid.

(*e*) To the first five tubes of each set add 0.5 c.c. of diluted antigen (carrying 10 antigenic units).

(*f*) After an interval of 5 to 30 minutes add 1 c.c. complement (2 full units) to each tube.

(*g*) Include the following controls:

Antigen control containing 0.5 c.c. diluted antigen, 0.5 c.c. salt solution and 1.0 c.c. diluted complement (2 full units).

Hemolytic system control containing 1.0 c.c. salt solution and 1.0 c.c. diluted complement (2 full units).

Corpuscle control containing 2.5 c.c. salt solution.

Positive and negative serum controls set up in dilutions as described above.

(*h*) Mix the contents of each tube by gently shaking and place in the refrigerator at 6° C. to 8° C. for 15 to 18 hours.

(*i*) Place tubes in the water bath at 38° C. for 5 to 15 minutes.

(*j*) To all tubes except the corpuscle control add 0.5 c.c. of hemolysin (carrying 2 units) and to all tubes add 0.5 c.c. of 2 per cent corpuscle suspension (shaken up).

(*k*) Mix the contents of each tube by gently shaking and place in the water bath at 38° C. for 1 hour.

(*l*) The table on page 285 shows the set-up for the quantitative complement fixation test.

After the secondary incubation place the tubes in the refrigerator for several hours to permit the settling of non-hemolyzed corpuscles. Read the degree of inhibition of hemolysis and record for each tube as—(complete hemolysis), + (25 per cent inhibition recorded as 1), + + (50 per cent inhibition recorded as 2), + + + (75 per cent inhibition recorded as 3), + + + + (100 per cent inhibition recorded as 4). All serum, antigen and hemolytic controls should show complete hemolysis. The corpuscle control should show no hemolysis.

TABLE XIII

Tube	Patient's Serum in 0.5 c.c.	Antigen 10 units	INTERVAL OF 5 TO 15 MINUTES AT ROOM TEMPERATURE.	Complement 2 full units	PRIMARY INCUBATION IN REFRIGERATOR AT 6° TO 8° C. FOR 15 TO 18 HOURS.	Hemolysin 2 units	Corpuscles 2 per cent	SECONDARY INCUBATION IN WATER AT 38° C. FOR 1 HOUR.
1	0.1 c.c.	0.5 c.c.		1.0 c.c.		0.5 c.c.	0.5 c.c.	
2	0.05 c.c.	"		"		"	"	
3	0.025 c.c.	"		"		"	"	
4	0.005 c.c.	"		"		"	"	
5	0.0025 c.c.	"		"		"	"	
6	0.1 c.c.	"		"		"	"	
7	Antigen Con. 0.5 c.c. Salt solution	"		"		"	"	
8	Hemol. Con. 1.0 c.c. Salt solution	—		"		"	"	
9	Corpuscle Con. 2.5 c.c. Salt solution	—		—		—	"	

Reactions may be interpreted as follows:

Very strongly positive: partial or complete fixation of complement in the first 4 or 5 tubes of the test. Occasionally a serum will show less fixation of complement in the first tube carrying 0.1 c.c. serum than in the second tube carrying 0.05 c.c.; examples of a very strongly positive reaction are 4431 – ; 44421 ; 1442 –.

Strongly positive: partial or complete fixation in the first 3 tubes. Examples: 441 – – ; 421 – – ; 241 – –.

Moderately positive: partial or complete fixation in the first 2 tubes. Examples: 43 – – – ; 31 – – – ; 12 – – –.

Weakly positive: partial or complete fixation is only the first tube. Examples: 4 – – – – ; 3 – – – – ; 1 – – – –.

Negative: complete hemolysis in all tubes, – – – – –.

The method of recording and reporting a complement fixation test by this method is as per the following example:

Quantitative Reaction	= Strongly positive (442 — —)
Serum 0.1 c.c.	= ++++
" 0.05 c.c.	= ++++
" 0.025 c.c.	= ++
" 0.005 c.c.	= —
" 0.0025 c.c.	= —
" 0.1 c.c. (control)	= —

Quantitative Reaction	= Moderately Positive (42 — — —)
Spinal fluid 0.5 c.c.	= ++++
" " 0.25 c.c.	= ++
" " 0.125 c.c.	= —
" " 0.0625 c.c.	= —
" " 0.03125 c.c.	= —
" " 0.5 (control)	= —

Technic of the New Kolmer Qualitative Complement Fixation Test for Syphilis

1. This test is conducted in exactly the same manner as described for the quantitative test except that two doses of serum (0.1 and 0.05 c.c. with 0.1 c.c. in the serum control) are employed. The 0.05 c.c. dose is included because sometimes the reaction is weaker and occasionally even negative with the 0.1 c.c. dose. With spinal fluid a single dose of 0.5 c.c. with 0.5 c.c. in the control is employed.

2. For each serum arrange 3 regulation tubes. Into No. 1 place 1.2 c.c. saline and 0.5 c.c. in Nos. 2 and 3.

3. Place 0.3 c.c. serum (heated 55° C. for 15 minutes) in No. 1; mix and transfer 0.5 c.c. to No. 2 and No. 3.

4. Mix No. 2 and discard 0.5 c.c.

5. For each spinal fluid arrange two tubes and place 0.5 c.c. (unheated) in each. The first tube receives antigen; the second does not and is the control.

6. Place 0.5 c.c. antigen (10 units) in Nos. 1 and 2 of each serum; also in control tube carrying 0.5 c.c. saline (antigen control).

7. Allow tubes to stand 5 to 30 minutes. Then add 2 full units of complement (0.5 c.c.) to each; also to a control carrying 0.5 c.c. saline (hemolytic system control).

8. Put up corpuscle control: 2.5 c.c. saline and 0.5 c.c. of 2 per cent suspension.

9. Mix tubes gently and place in refrigerator at 6° to 8° C. for 16 to 18 hours. Keep hemolysin and corpuscle suspension in refrigerator.

10. Place tubes in water bath at 38° C. for 5 to 10 minutes (not longer).

11. To all tubes except corpuscle control add 2 units of hemolysin.

12. To all tubes except corpuscle control add 0.5 c.c. of 2 per cent corpuscle suspension (shaken up).

13. Mix and water bath for 1 hour. Refrigerator for hour or two.

14. The serum and hemolytic controls should be completely hemolyzed; the corpuscle control should show no hemolysis.

15. Read and record the serum tests by the —, + (1), + + (2), + + + (3), and + + + + (4) plan as follows:

+ + + + in either or both tubes = very strongly positive
+ + + in either or both tubes = strongly positive
+ + in either or both tubes = moderately positive
+ in either or both tubes = weakly positive
— in both tubes = negative

16. Read spinal fluid tests as follows:

+ + + + = very strongly positive
+ + + = strongly positive
+ + = moderately positive
+ = weakly positive
— = negative

Technic of the Kolmer Gonococcus, Typhoid, Tuberculosis and Echinococcus Complement Fixation Tests

1. These are conducted by the quantitative or qualitative methods exactly as described except that the respective antigens are employed.

2. The anticomplementary unit of the antigen is determined by titration in exactly the same manner as described on page 277 except that water bath incubation of 1 hour at 38° C. is employed instead of 16 to 18 hours in a refrigerator in order that the anti-

complementary unit may be determined each time the tests are conducted since the bacterial antigens do not keep well.

3. The antigen is used in ¼ its anticomplementary unit so diluted as to be carried in 0.5 c.c.

4. The tests are now set up in the same manner as described including antigen, hemolytic and corpuscle controls. Primary incubation is 16 to 18 hours in refrigerator at 6° to 8° C. followed by 10 minutes in water bath, etc.

5. The reactions are recorded in the same manner as described.

6. Bacterial antigens are usually troublesome to prepare and may be purchased. A good *gonococcus antigen* is made by suspending 6 or more different strains in saline, heating to 60° C. for one hour and preserving with 0.5 per cent phenol. A good cloudy suspension is required. *Typhoid antigen* may be prepared in the same manner, using several strains if possible. *Tuberculosis antigen* should be prepared of degreased bacilli, dried and thoroughly pulverized. To 19 c.c. of distilled water add 0.1 gm. and boil with a reflux condenser for one hour. Add 1 c.c. of 20 per cent sodium chlorid carrying 5 per cent phenol to render antigen isotonic and for preservation. *Echinococcus antigen* is the cyst fluid preserved with 0.5 per cent phenol. It should be heated at 60° C. for thirty minutes to reduce anticomplementary activity, just before titration and use.

Technic of the Kahn Precipitation Serum Test for Syphilis

Preparation of Antigen

Powdered beef heart, prepared by the Digestive Ferments Company of Detroit, may be used.

Twenty-five gm. of powdered beef heart are placed in a 250 c.c. Erlenmeyer flask and 100 c.c. ether (anesthesia) added. The flask is shaken from time to time for an interval of ten minutes after which the ether is filtered off.

The moist beef heart is now returned to the original 250 c.c. extraction flask. Seventy-five c.c. ether are now added and the mixture again shaken for a ten-minute interval and filtered as above.

After the second filtration of ether, the beef heart is transferred to the flask for a third time and 75 c.c. ether again added. The

mixture is shaken for a ten-minute interval and again filtered as described above.

The moist beef heart is now transferred for the fourth and last time to the flask and 75 c.c. ether added, the mixture shaken for ten minutes and filtered. After gentle pressure with a spatula does not cause drops of ether to pass through the funnel—the endpoint employed in each of the four ether filtrations—the beef heart is transferred to a sheet of white paper and dried either at room temperature or at 37° C. The drying usually requires from ten to fifteen minutes. When no ether odor is detectable, the beef heart is ready for extraction with alcohol. This extraction may be carried out in the same flask used for the ether extractions, provided the flask is entirely freed from ether odor.

The dry powdered muscle is weighed and placed in a 250 c.c. flask. Usually there will be 23 gm. or less of the powder due to the loss during the ether extraction. Five c.c. of 95 per cent alcohol are added per gm. of powder. The flask is shaken for ten minutes and extraction allowed to continue at room temperature (21° C.) for three days without shaking. At the end of this period the mixture is shaken for five minutes and filtered. The filtrate is kept in the dark at room temperature as stock antigen solution.

A given amount of alcoholic extract—likely to be used in a month or two—is cholesterinized by adding 6 mg. of chemically pure cholesterin per c.c. of extract. The cholesterin is dissolved by rotating the flask in a water bath at 37° C. When all the cholesterin has been dissolved, the antigen is filtered to remove impurities and allowed to stand one day. It is then ready to be titrated or standardized for the test.

Method of Antigen Titration

One c.c. of cholesterinized antigen is added to each of five standard antigen dilution tubes (5.5 cm. length and 1.5 cm. diameter). To five similar tubes are added the following amounts of physiologic salt solution, respectively: 0.8, 0.9, 1.0, 1.1 and 1.2 c.c. Each salt solution tube is emptied into a given antigen tube and, without waiting to drain the salt solution, the mixture is immediately poured back and forth five or six times to permit thorough mixing. Each of the five antigen dilutions will show

the presence of a definite precipitate. The character of these precipitates, however, will vary according to the quantity of salt solution used. Some of the precipitates will be found to be stable and will not dissolve when mixed with salt solution; other precipitates will readily dissolve in salt solution.

The solubility in salt solution of each of the five antigen dilution precipitates is tested as follows: 0.05, 0.025 and 0.0125 c.c. amounts, respectively, of each antigen dilution are pipetted with a 0.2 c.c. pipet graduated in 0.001 c.c. into three tubes (7.5 cm. in length and 1 cm. in diameter). These small quantities are pipetted in each case to the bottom of the tubes. 0.15 c.c. quantities of physiologic salt solution are now added to each tube. The rack is shaken vigorously for two minutes after which 0.5 c.c. salt solution is added to each tube and observation made as to whether or not the original antigen dilution precipitate has gone back into solution. The antigen dilution tube containing the smallest amount of salt solution in proportion to antigen, having a precipitate which goes back into solution in salt solution, as shown by this three tube test, represents the endpoint of this titration and determines the proportion in which antigen is to be mixed with salt solution in the performance of the tests.

The Serum

The serums are separated from the clots by centrifugation in the usual manner, pipetted off and inactivated for 30 minutes at 56° C.

Physiologic Salt Solution

Salt solution is prepared by dissolving 8.5 gm. of chemically pure sodium chlorid per liter of distilled water and filtering.

The Routine Test with Serum

(*a*) The routine test consists of three tubes containing three different proportions of serum and antigen dilution in accordance with the following outline:

TABLE XIV

Tube Number	1	2	3
Serum: antigen dilution	3:1	6:1	12:1
Antigen dilution c.c.	.05	.025	.0125
Serum c.c.	.15	.15	.15

(*b*) One c.c. antigen is measured into an antigen dilution tube. An amount of salt solution usually approximating that of the antigen, according to the titer of the antigen, is measured into a similar tube. The salt solution is poured into the antigen and, without waiting to drain the tube, the mixture is immediately poured back and forth five or six times to insure thorough mixing. For uniformity, this antigen dilution is permitted to stand ten minutes at room temperature before pipetting.

As a preliminary control of the antigen dilution, 0.05 c.c. are measured into a test tube, 1 c.c. salt solution added and the tube shaken vigorously for ten or fifteen seconds. The mixture should appear opalescent.

(*c*) The antigen dilution is always pipetted to the bottom of the tubes. The 0.05 c.c. amounts may be pipetted with a 1 c.c. pipet in which 0.05 c.c. graduations are indicated with a wax pencil. For the 0.025 and 0.0125 c.c. amounts of antigen dilution, 0.2 c.c. pipets are employed and the proper markings may also be indicated with a wax pencil. The antigen dilution should be mixed frequently during the pipetting period to assure a uniform mixture. Due to the possibility of evaporation of the small amounts of antigen dilution used in the test, it is necessary to pipet the serum within several minutes after the antigen dilution has been pipetted. If a worker desires to run forty precipitation tests, for example, it is better to pipet antigen dilution and serums for ten tests at a time than to pipet the antigen dilution for all the tests first and then follow with the serums.

(*d*) The 0.15 c.c. amounts of each serum are added to the antigen dilution by means of a 1 c.c. pipet graduated in 0.01 c.c. In pipetting these amounts of serum it is not necessary to lower the pipet to the bottom of the tube. As soon as the serums have been added for ten tests, or less, the rack is shaken sufficiently to insure thorough mixing of the serum with antigen dilution.

(*e*) After the serums have been mixed with the antigen dilution, the tests are shaken for a two-minute interval. A shaking machine is of the utmost importance for this purpose, particularly in the examination of comparatively large numbers of specimens at a given time.

(*f*) Although the final results may be read immediately after the shaking period, different workers have observed that a 15-

minute incubation period in the water bath at 37° C. produces sufficient clumping of the precipitates to make the reading of the results easier, particularly in the case of weak reactions.

(*g*) After the shaking of the tests, as well as after the 15-minute incubation period, if employed, the serum-antigen mixtures appear uniformly cloudy. In order to render the negative reactions clear and thus simplify the reading of results, 0.5 c.c. salt solution is added to each tube. The tests should be read immediately after the addition of salt solution as an occasional weak precipitate may go back into solution on standing.

Readings are best made in front of a window with a darkened background. The negative serums appear opalescent and readily distinguishable without lifting the tubes from the racks. The strongly positive serums show heavy precipitates which are also easily read directly.

Only the tubes showing weak reactions need to be removed from the rack and examined individually. Each tube is lifted from the rack above the eye level, slanted to spread the fluid into a thin layer and examined for a precipitate.

(*h*) *Antigen Control.*—When pipetting antigen dilution for a series of tests, the last set-up of three regular antigen amounts receives 0.15 c.c. salt solution instead of serum and is read with the regular tests. All three tubes should show freedom from a precipitate.

Serum Controls.—In the case of each positive reaction, the serum used in the test is examined for particles that might give the appearance of a specific precipitate.

Positive and Negative Controls.—One or more such controls are included with each series of tests.

(*i*) A definite precipitate suspended in a clear medium is considered a complete reaction and is read four plus. Proportionally weaker reactions are read three, two and one plus, respectively. The final result in each test is the average finding of the three tubes. Thus, if the precipitation reaction is four plus in each of the three tubes, the final result is four plus. If the reaction is −, + + +, + + + +, the final result is two plus.

SECTION IV

BLOOD CHEMISTRY

SECTION IV

CLINICAL BLOOD CHEMISTRY METHODS

Method of Obtaining Blood

1. The patient should not have eaten within three hours of the drawing of blood.

2. A test tube carrying 0.03 to 0.05 gm. of potassium or lithium oxalate [1] and stoppered with cork or rubber is required; also a sterilized 25 c.c. Record or Luer syringe with a needle of about gauge 18.

3. Place a tourniquet firmly about the arm above the elbow; request the patient to open and close the hand quickly and firmly which aids in distending the superficial veins. Cleanse the skin with alcohol.

4. Assemble the syringe and withdraw 15 to 25 c.c. of blood: for sugar or urea determinations alone, 5 c.c. will suffice; for complete Folin-Wu analysis of non-protein nitrogen, urea, creatinin, creatin, uric acid and sugar about 15 c.c. are required; for cholesterol add 5 c.c.

5. Place blood at once in the oxalate tube; stopper and gently mix for a minute or two to prevent coagulation.

6. If plasma is required it is obtained by centrifuging.

Preparation of Protein Free Blood Filtrate for the Folin and Wu Analyses

1. Transfer a measured quantity (5 to 15 c.c.) of oxalated blood to a small Erlenmeyer flask (5 c.c. of blood sufficient for

[1] Tubes are prepared by placing in each 3 c.c. of a 10 per cent solution of potassium oxalate, stoppering with cotton and drying in a hot air oven which leaves the inside of the tube coated with oxalate. An excess is to be avoided in urea determinations in order not to interfere with deproteinization; otherwise a slight excess is not harmful.

sugar or urea; 15 c.c. for complete analysis for non-protein nitrogen, urea, creatinin, sugar, etc.). Add seven volumes of water and lake the blood; add one volume of a 10 per cent solution of sodium tungstate (made up from the C. P. sodium tungstate of the J. T. Baker Chemical Company) and mix.

2. Add from a graduated pipet or buret, very slowly (drop by drop) and with constant shaking, one volume of two-thirds normal sulphuric acid (35 gm. C.P. sulphuric acid to about 700 c.c. of water, cool, add water to exactly 1000 c.c. Usually correct, but should be checked up by titration). Close the mouth of the flask with a rubber stopper and shake.

3. Let stand 5 minutes (15 minutes if uric acid is to be determined); the color changes to dark brown; if not, coagulation is incomplete in which case add 10 per cent sulphuric acid drop by drop until there is practically no foaming and a dark brown color results.

4. Pour a few cubic centimeters on a filter with double papers large enough to hold all; after the whole filter is wet add the balance and cover with a watch glass.

5. The filtrate should be used at once; if it is to be kept for several days add a few drops of toluene and place in a refrigerator.

Estimation of Non-protein Nitrogen (Folin and Wu)

1. Transfer 5 c.c. of the blood filtrate to a large dry pyrex test tube (200 mm. x 25 mm.) preferably graduated at 35 c.c. and 50 c.c.

2. Add 1 c.c. of diluted phosphoric sulphuric acid mixture (1 part acid mixture diluted with equal part of water) and a quartz pebble.

3. Boil vigorously over a microburner for 3 to 7 minutes until dense fumes fill the tube. Then lower the flame to reduce boiling almost to the vanishing point, cover the tube with a watch glass and continue gentle heating for 2 more minutes or until the solution is nearly colorless (usually colorless after 20 to 40 seconds).

4. At the end of the 2 minutes remove flame and allow solution to cool for 70 to 90 seconds and add water to the 35 c.c. mark.

5. Add 15 c.c. of Nessler's solution (up to the 50 c.c. mark), insert a rubber stopper and mix. If the solution is turbid, centrifuge a portion before making the color comparison.

6. Place 3 c.c. of standard ammonium sulphate solution in a 100 c.c. volumetric flask. Add 2 c.c. of the phosphoric sulphuric acid mixture, about 50 c.c. of water, and 30 c.c. of Nessler solution. Fill to the 100 c.c. mark and mix.

7. Set the standard at 20 mm. for the color comparison. Make the reading.

8. The non-protein nitrogen per 100 c.c. of blood is 20 divided by the reading and multiplied by 30 (0.3 times 100).

Phosphoric Sulphuric Acid Mixture:—To 50 c.c. of a 5 per cent copper sulphate solution add 300 c.c. of 85 per cent phosphoric acid and mix. Add 100 c.c. of concentrated sulphuric acid free from the least trace of ammonia and mix. Keep well stoppered to prevent absorption of ammonia from the air.

Standard Ammonium Sulphate Solution:—Dissolve 0.283 gm. of pure ammonium sulphate in 1000 c.c. of distilled water.

Nessler Reagent (Folin Modification):—In a 500 c.c. Florence flask place 150 gm. of potassium iodid, 110 gm. of iodin, add 100 c.c. of water and 150 gm. of metallic mercury. Shake continuously for 7 to 15 minutes, or until the iodin has nearly disappeared. When the red solution pales, cool in running water and continue shaking until solution is greenish. Decant and wash mercury with liberal quantities of distilled water, diluting the solution and washings to 2000 c.c. Introduce into a 5 liter flask 750 c.c. of this stock solution, add 3500 c.c. of 10 per cent sodium hydroxid solution and 750 c.c. of distilled water. The alkalinity of the resulting Nessler solution is important and may be checked by titration.

Estimation of Urea

Method of Folin and Wu

1. Place 5 c.c. of the filtrate in a Pyrex ignition tube (200x25m.). If tube has contained Nessler solution first rinse with nitric acid and then with water before using.

2. Add 2 drops of buffer mixture (made by dissolving 69 gm. of monosodium phosphate and 179 gm. of crystallized disodium phosphate in 800 c.c. of warm distilled water and diluting to 1000 c.c.).

3. Add 1 c.c. of urease solution prepared as follows: Wash 3 gm. of permutit in a flask, once with 2 per cent acetic acid, then twice with water; add 5 gm. of fine jack bean meal and 100 c.c.

of 15 per cent alcohol. Shake gently for 15 minutes, pour on large filter, and cover with a watch glass. The filtrate may be kept 4 to 6 weeks in an ice box.

4. Mix and immerse the tube in a water bath at 40° to 55° C. for 5 minutes, or let stand at room temperature for 15 minutes.

5. Add a dry pebble, 2 c.c. of saturated borax solution and a drop or two of paraffin oil.

6. Distil off the ammonia by inserting a rubber stopper carrying a delivery tube to the bottom of a second test tube marked at 25 c.c. and containing 2 c.c. of normal hydrochloric acid, and by boiling moderately fast for 4 minutes.

7. At the end of 4 minutes slip off the rubber stopper from the receiving tube and let the delivery tube rest in a slanting position above the hydrochloric acid. Boil for 1 more minute and rinse off the lower end of the delivery tube with a little distilled water.

8. Cool the distillate in running water and dilute to about 20 c.c. Add 2.5 c.c. of Nessler solution (see page 307) and fill with water to the 25 c.c. mark.

9. Compare in a colorimeter with a standard, prepared by placing 3 c.c. of the standard ammonium sulphate solution (see page 307) in a 100 c.c. flask, adding 75 c.c. of water, 10 c.c. of Nessler solution and water to 100 c.c.

10. Divide 20 (the height of the standard in mm.) by the reading and multiply by 15 to obtain the urea nitrogen in mgs. per 100 c.c. of blood.

Van Slyke and Cullen's Modification of Marshall's Method

1. Place 1 c.c. of a 3 per cent solution of potassium citrate in a test tube and add 3 c.c. of fresh blood obtained by vein puncture.

2. Add 0.5 c.c. of urease solution and 2 or 3 drops of caprylic alcohol.

3. Wait 10 minutes and add 15 c.c. of a saturated solution of potassium carbonate.

4. Aspirate the ammonia into another tube containing 15 c.c. of n/100 hydrochloric acid.

5. Titrate the excess acid with n/100 sodium hydroxid using methyl red as indicator.

6. Each cubic centimeter of acid neutralized by the ammonia during aspiration indicates 0.01 gm. of urea per 100 c.c. of blood, or 0.00467 grain of urea nitrogen per 100 c.c. of blood.

Folin Urease Solution.—Place 3 gm. of permutit in a flask and wash once with 2 per cent acetic acid and twice with water. Add 5 gm. of fine Jack bean meal and 100 c.c. of 15 per cent alcohol (16 c.c. of 95 per cent mixed with 84 c.c. of water). Shake gently but continuously for 10 to 15 minutes and filter. Keep in ice box for 4 to 6 weeks. Concentrated preparations of Jack bean urease containing the activating phosphates may be obtained on the market.

Estimation of Preformed Creatinin (Folin and Wu)

1. Prepare alkaline picrate solution by placing 25 (or 50) c.c. of a saturated solution of purified picric acid in a small, clean flask; add 5 (or 10) c.c. of 10 per cent solution of sodium hydroxid and mix.

2. In a second small flask or test tube place 10 c.c. of blood filtrate and 5 c.c. of the alkaline picrate solution.

3. In a third small flask or test tube place 5 c.c. of standard creatinin solution, 15 c.c. of distilled water and 10 c.c. of the alkaline picrate solution.

4. Let stand for 8 to 10 minutes and make the color comparison in a colorimeter in the usual manner, first making sure that the two fields of the colorimeter are equal when both cups contain standard creatinin picrate solution. The color comparison should be completed within 15 minutes from the time the tests are set up.

5. The reading of the standard in mm. (usually 20) multiplied by 1.5, 3, 4.5, or 6 (according to how much of the standard solution was taken), and divided by the reading of the unknown in mm., gives the amount of creatinin in mg. per 100 c.c. of blood.

Standard Creatinin Solution.—Prepare a stock solution of creatinin by dissolving 0.1 gm. in 50 c.c. of water, adding 1 c.c. of concentrated hydrochloric acid and water up to 100 c.c.

In a liter flask place 6 c.c. of this stock solution, 1 c.c. of pure concentrated hydrochloric acid and water to 1000 c.c. Mix, transfer to a bottle and add four or five drops of toluene or xylene. Five c.c. of this solution contain 0.03 mg. of creatinin, and this amount plus 15 c.c. of water represents the standard required for most human bloods (1 to 2 mg. per 100 c.c.). In the case of unusual bloods showing a retention of creatinin, take 10 c.c. of the standard plus 10 c.c. of water, which covers the range of 2 to 4

mg. per 100 c.c. of blood; 15 c.c. plus 5 c.c. of water corresponds to 4 to 6 mg. per 100 c.c. of blood; the full 20 c.c. of standard solution corresponds to 8 mg. per 100 c.c. of blood.

Estimation of Total Creatinin (Creatin Plus Creatinin) Folin and Wu

1. Place 5 c.c. of blood filtrate in a test tube graduated at 25 c.c. Add 1 c.c. of normal hydrochloric acid.

2. Cover the mouth of the tube with tin foil and heat in an autoclave to 130° C. for 20 minutes, or to 155° C. for 10 minutes.

3. Cool, add 5 c.c. of alkaline picrate solution (see page 311) and let stand 8 to 10 minutes; dilute to 25 c.c. with distilled water.

4. In the meantime prepare the standard solution by placing 2 c.c. of normal hydrochloric acid in a volumetric flask, adding 10 c.c. of alkaline picrate solution, and, after standing 10 minutes, water to 50 c.c.

5. Make readings in a colorimeter. The height of the standard (usually 20 mm.) divided by the reading of the unknown and multiplied by 6 gives the total creatinin in mg. 100 c.c. blood.

Estimation of Creatin

1. Determine the preformed creatin as described.

2. Determine the total creatin as described.

3. Subtract the preformed creatin from the total creatinin and multiply by 1.16 which gives mg. of creatin per 100 c.c. blood.

Estimation of Uric Acid

Method of Folin and Wu

1. Fill a pyrex 1 liter beaker two-thirds full with water and heat to boiling.

2. In a test tube graduated at 25 c.c. place 5 c.c. of blood filtrate and 2 c.c. of water.

3. In a second similar test tube place 5 c.c. of diluted standard uric acid solution and 2 c.c. of water.

4. To both tubes add 3 drops of 20 per cent lithium sulphate solution, 2 c.c. of 15 per cent sodium cyanid solution and 1 c.c. of the standard uric acid reagent.

5. Mix each tube, let them stand for 2 minutes and then place both in the boiling water for 80 seconds.

6. Cool and add water to each to 25 c.c. mark.

7. Make the color comparison in the usual manner, first reading the standard against itself.

8. When the standard is set at 20 mm., 20 divided by the reading of the unknown, times 4, gives the uric acid content in milligrams per 100 c.c. of blood.

9. If the uric acid content is too high (reading less than 10 mm.) repeat with 3 c.c. of blood filtrate (plus 2 c.c. of water). If too weak, repeat with 10 c.c. of filtrate and 5 c.c. of the standard (plus 5 c.c. of water). Make necessary alterations in the calculation accordingly.

Standard Uric Acid Solution.—Place 0.1 gm. of pure uric acid in a clean 100 c.c. beaker. Dissolve 0.05 gm. of lithium carbonate in 20 c.c. of water in a small beaker and heat to 60° to 70° C., and pour this warm alkali on the uric acid and stir until it is completely dissolved. As soon as a clear solution is obtained, add 5 c.c. of 40 per cent formaldehyd and 1 c.c. of 50 per cent acetic acid. Transfer to a 100 c.c. volumetric flask and dilute with water to the mark; transfer to a bottle. This solution contains 1 mg. of uric acid per cubic centimeter and will keep several months.

The diluted uric acid standard containing 0.02 mg. of uric acid in 5 c.c. is prepared by placing 1 c.c. of the standard solution in a 250 c.c. volumetric flask, half filling with water, adding 10 c.c. of ⅔ normal sulphuric acid as used in blood protein precipitation, 1 c.c. (but no more) of 40 per cent formaldehyd and water to the 250 c.c. mark. Keep at least 1 month.

Lithium Sulphate Solution.—Dissolve 20 gm. of Baker and Adamson's powdered lithium sulphate in about 80 c.c. of cold water. Dilute to 100 c.c. in a volumetric flask and filter.

Sodium Cyanid Solution.—Weigh out on paper 100 gm. of fresh sodium cyanid and transfer to a beaker; add 670 c.c. of decinormal sodium hydroxid solution and dissolve. Transfer to a bottle and let stand two weeks before using.

Uric Acid Reagent.—Introduce into a flask 750 c.c. of water, 100 gm. of sodium tungstate and 80 c.c. of phosphoric acid (85 per cent H_3PO_4). Partly close the mouth of the flask with a funnel and small watch glass, and boil gently for two hours. Dilute to a liter.

Benedict's Method

1. Place 5 c.c. of blood filtrate in a test tube of 18 to 20 mm. in diameter and add 5 c.c. of water.

2. In a second tube place 5 c.c. of dilute standard uric acid solution containing 0.02 mg. of uric acid; add 5 c.c. of water.

3. To both tubes add 4 c.c. of 5 per cent sodium cyanid-ammonia and 1 c.c. of arsenic phosphoric acid tungstic acid reagent.

4. Mix the contents of each tube by one immersion and place at once in boiling water for 3 minutes.

5. Remove tubes and place them in cold water for 3 minutes and then make colorimetric readings at once.

6. The reading of the standard, divided by the reading of the blood filtrate, multiplied by 4, gives mg. of uric acid per 100 c.c. of blood.

Sodium Cyanid Ammonia Solution.—Dissolve 50 gm. of sodium chlorid in 700 c.c. of water; add 2 c.c. of concentrated ammonia and water to make 1000 c.c. Prepare fresh every two months.

Arsenic Phosphoric Acid-Tungstic Acid Reagent.—Place 100 gm. of pure sodium tungstate in a liter flask and add 600 c.c. of water. Add 50 gm. of pure arsenic pentoxid followed by 25 c.c. of 85 per cent phosphoric acid, and 20 c.c. of concentrated hydrochloric acid. Boil the mixture for 20 minutes, cool and dilute to 1 liter. Keeps indefinitely.

Stock Uric Acid Standard Solutions.—Dissolve 9 gm. of pure crystallized disodium hydrogen phosphate and 1 gm. crystallized sodium dihydrogen phosphate in 200 to 300 c.c. of hot water. Filter if not perfectly clear. Make up to about 500 c.c. with hot water and pour upon exactly 200 mg. pure uric acid suspended in a few c.c. of water in a liter volumetric flask. Agitate until uric acid is dissolved. Cool, add *exactly* 1.4 c.c. of glacial acetic acid, dilute to mark and mix. Add about 5 c.c. of chloroform to prevent contamination. Solution contains 1 mg. of uric acid per 5 c.c. and keeps about 2 months.

Dilute Uric Acid Standard Solution.—In a 500 c.c. volumetric flask place 5 c.c. of the stock solution and half fill with water; add 25 c.c. of dilute hydrochloric acid (1 volume of concentrated acid diluted to 10 volumes with water) and water to the 500 c.c. mark. Each 5 c.c. contains 0.02 mg. uric acid; solution keeps 2 weeks.

Method for Estimating Sugar (Folin and Wu)

1. In a Folin-Wu sugar tube place 2 c.c. of the blood filtrate and add 2 c.c. of alkaline copper solution.

2. In two additional tubes place 2 c.c. of standard sugar solutions containing, respectively, 0.2 and 0.4 mg. of glucose. To each tube add 2 c.c. of alkaline copper solution.

3. The surface of the mixtures must reach the constricted part of each of the three tubes. If the bulb is too large for the volume (4 c.c.) not more than 0.5 c.c. of diluted (1:1) alkaline copper solution may be added.

4. Place the three tubes in boiling water for 6 minutes.

5. Transfer to cold water without shaking for 3 minutes.

6. To each tube add 2 c.c. of the molybdate phosphate solution and, after dissolving the cuprous oxid in each, add water to the 25 c.c. mark.

7. Insert a rubber stopper in each tube and mix thoroughly.

8. Take readings with colorimeter. The depth of the standard in millimeters (20) multiplied by 100, or by 200 if the stronger standard is taken, and divided by the colorimetric reading of the blood gives the sugar content in mg. per 100 c.c. of blood.

Alkaline Copper Solution.—Dissolve 40 gm. of anhydrous sodium carbonate in 400 c.c. of water and transfer to a volumetric liter flask. Add 7.5 gm. of tartaric acid, and, when this has dissolved, add 4.5 gm. of crystallized copper sulphate. Dissolve and add water to 1 liter.

Standard Sugar Solutions.—Dissolve 2.5 gm. of benzoic acid in 1 liter of boiling water and cool. This is a stock 0.3 per cent solution and keeps indefinitely.

Dissolve 1 gm. of pure glucose in 100 c.c. of stock benzoic acid solution. This 1 per cent glucose solution keeps indefinitely.

Dilute 5 c.c. of stock glucose solution to 500 c.c. with stock benzoic acid solution; this solution contains 0.1 mg. of glucose per c.c.

Dilute 5 c.c. of stock glucose solution to 250 c.c. with stock benzoic acid solution; this solution contains 0.2 mg. of glucose per c.c.

Molybdate Phosphate Solution.—In a liter beaker place 35 gm.

of molybdic acid, 5 gm. of sodium tungstate, 200 c.c. of 10 per cent sodium hydroxid and 200 c.c. of water. Boil vigorously for 20 to 40 minutes, cool, dilute to about 350 c.c., add 125 c.c. of concentrated (85 per cent) phosphoric acid and dilute with water to exactly 500 c.c.

Method of Conducting Carbohydrate Tolerance Test (Killian)

1. Give first thing in the morning a standard breakfast of two slices of bread, one egg in any form and one cup of water.

2. Two hours later have patient urinate and drink 200 c.c. of water.

3. One hour later collect specimen of blood and urine as controls.

4. Then give patient 1.75 gm. of glucose per each kilo of body weight (approximately 2 pounds) in the form of a 50 per cent solution in water.

5. Collect 3 or 4 specimens of blood at hourly intervals.

6. Determine sugar content of the specimens of blood including the control.

7. Collect a 24-hour specimen of urine and determine glucose content quantitatively of this urine and the control specimen.

8. In normal individuals the blood sugar rises from the normal value of about 100 mg. per 100 c.c. of blood to about 150 mg. at the end of the first hour, and returns to normal by the end of the second hourly period. High sugar concentration in the blood may or may not be accompanied by glycosuria depending upon the "threshold point" of the kidney.

The test may be conducted without the preliminary standard meal. Two or three hours after last meal take a specimen of blood for a control; now give same amount of glucose per kilo of weight in 40 per cent solution together with the juice of one lemon; one and two hours later draw blood. Analyze the three specimens for sugar.

Estimation of Chlorids (Whitehorn-Folin)

1. Place 10 c.c. of protein free (tungstic acid) filtrate (equivalent of 1 c.c. of blood) in a porcelain dish.

2. Add 5 c.c. of standard silver nitrate solution and mix thoroughly.

3. Then add 5 c.c. of concentrated nitric acid (sp. gr. 1.42) and let stand 5 minutes.

4. Add about 0.3 gm. of powdered ferric ammonium sulphate with a spatula.

5. Titrate the excess of silver nitrate with standard sulphocyanate solution until the definite salmon-red (not yellow) color of the ferric sulphocyanate persists in spite of stirring for at least 15 seconds.

6. 5 c.c. (standard $AgNO_3$ solution)—titer in c.c. gives mg. of Cl per c.c. of blood (or plasma). To express as sodium chlorid multiply Cl value by 1.65.

Standard Silver Nitrate Solution.—Dissolve 4.791 gm. of C.P. silver nitrate in 1000 c.c. of distilled water (1 c.c. contains 1 mg. Cl).

Standard Sulphocyanate Solution.—Dissolve 3 gm. of potassium sulphocyanate in a liter of distilled water. Titrate under conditions described in the method and dilute so that 5 c.c. are equivalent to 5 c.c. of the silver nitrate solution.

Estimation of Cholesterol (Myers and Wardell)

1. Place 1 c.c. of blood, plasma or serum into a small beaker and add 4 to 5 gm. of plaster of Paris.

2. Stir thoroughly and dry in an oven for one hour.

3. Empty into a paper extraction shell (4 cm. long) and insert in a short glass tube (2.5 x 7 cm.) in the bottom and sides of which are a number of small holes.

4. Attach to a large cork carrying a small reflux condenser and insert in the neck of a 150 c.c. extraction flask containing about 25 c.c. of chloroform.

5. Extract on an electric hot plate for 90 minutes.

6. Add chloroform to make 20 c.c. and filter if necessary.

7. Place 5 c.c. of the chloroform extract in a dry test tube; add 2 c.c. of acetic anhydrid and 0.1 c.c. of concentrated sulphuric acid. Mix thoroughly and place in a dark cool place for exactly 10 minutes.

8. Compare with a standardized 0.005 per cent water solution of naphthol green B in a Bock-Benedict or Klett colorimeter (if the Duboscq is used the cup should be remounted in plaster of Paris instead of balsam). Or compare with a cholesterol standard con-

taining 0.4 mg. of cholesterol in 5 c.c. of chloroform treated with 2 c.c. of acetic anhydrid and 0.1 c.c. of concentrated sulphuric acid in a Duboscq or Klett instrument.

9. $\frac{S}{R} \times 0.0004 \times \frac{D}{5} \times 100 =$ cholesterol content of the blood

in per cent, in which S stands for the depth of standard in mm., R for the reading of the blood being tested, 0.0004 the equivalent amount of cholesterol in 5 c.c. of chloroform, D the dilution of the chloroform extract from the 1 c.c. of blood, 5 the dilution of the standard, and 100 the factor for 100 c.c. For example:

$\frac{15}{15} \times 0.0004 \times \frac{20}{5} \times 100 = 0.160$ per cent.

TABLE XV

	Normal *	Chronic Nephritis	Uremia	Early Diabetes	Severe Diabetes
Non-Protein N...	25 to 30	30 to 90	90 to 400		
Urea N...	12 to 15	16 to 70	70 to 300		
Uric acid..	2 to 3.5	3 to 10	4 to 25		
Creatinin..	1 to 2	2 to 4	4 to 35		
Creatin ...	3 to 7		7 to 30		
Sugar, per cent	0.08 to 0.12		0.1 to 0.2	0.14 to 0.30	0.3 to 1.2
Cholesterol	140 to 180	170 to 350	170 to 350		150 to 300
Chlorids ..	0.65	0.55 to 0.75	0.45 to 0.65		0.60

* Expressed as mg. per 100 c.c. of blood unless otherwise indicated.

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